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# Genetic Variability and Geographic Diversity of the Common Bed Bug (Hemiptera: Cimicidae) Populations from the Midwest Using Microsatellite Markers

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## Abstract

With the recent global resurgence of the bed bugs (*Cimex lectularius* L.), there is a need to better understand its biology, ecology, and ability to establish populations. Bed bugs are domestic pests that feed mainly on mammalian blood. Although bed bugs have not been implicated as vectors of pathogens, their biting activity inflicts severe insomnia and allergic reactions. Moreover, they have recently developed resistance to various insecticides, which requires further molecular research to determine genetic variation and appropriate interventions. Population dynamics, including genetic differentiation and genetic distance of 10 populations from the Midwest were analyzed in this study. The bed bug samples collected by pest control companies were genotyped using eight species-specific microsatellite markers. Results showed all eight markers were polymorphic, with 8–16 alleles per locus, suggesting high genetic diversity. The  $F_{ST}$  values were  $>0.25$ , signifying pronounced genetic differentiation. The G-test results also indicated high genetic differentiation among populations. The frequency of the most common allele across all eight loci was 0.42. The coefficient of relatedness between each of the populations was  $>0.5$ , indicative of sibling or parent-offspring relationships, while the  $F_{IS}$  and its confidence interval values were statistically insignificant within the populations tested. The populations departed from Hardy-Weinberg equilibrium, possibly because of high heterozygosity. The genetic distance analysis using a neighbor-joining tree showed that the populations from Kansas City, Missouri, were genetically separate from most of those from Nebraska, indicating

a geographic pattern of genetic structure. Our study demonstrated the effectiveness of using microsatellite markers to study bed bug population structure, thereby improving our understanding of bed bug population dynamics in the Midwest. Overall, this study showed a high genetic diversity and identified several new alleles in the bed bug populations in the Midwest.

**Keywords:** bed bug, genetic analysis, genetic diversity, microsatellite marker

Recent reports of worldwide outbreaks and rapid infestations of the bed bug (*Cimex lectularius* L.) demand attention to better the understanding of their biology, population composition, and breeding structure. Of the 92 described cimicids, 16 species are commonly found in United States and Canada (Usinger 1966, Snetsinger 1997). Bed bugs are intermittent ectoparasites that harbor in cracks and crevices of structures and feed exclusively on the blood of humans, chimpanzees, monkeys, bats, and birds (Harraca et al. 2012). The prevalence of bed bugs in human abodes has been reported for at least four millennia (Seidel and Reinhardt 2013); however, studies indicated a steady decline of bed bugs in developed countries from 1930 until 1990 (Boase 2008, Potter 2008). The resurgence of bed bugs in the United States occurred during the mid-1990s, with complaints to pest control operators increasing on average 25% per year. (Davies et al. 2012, Doggett et al. 2012). Although several explanations (increased international travel, ban of dichlorodiphenyltrichloroethane, and change in insecticide applications for cockroach control from residual insecticide to baiting) have been proposed to account for the resurgence, none of them were conclusive (Doggett et al. 2004, Romero et al. 2007, Boase 2008, Bencheton et al. 2010).

Anderson and Leffler (2008) have surveyed news articles from 2001 to 2006 and concluded that bed bugs are a great concern in all 50 states by relating the number of articles to the bed bug prevalence in each state. According to that survey, the Northeast region, mainly New York and Washington, DC, ranked first, and the Midwest was the last to show widespread bed bug infestations. A follow-up survey in 2011 (Potter et al. 2011) showed a >20% increase in all parts of the United States. To address concerns from the public, the U.S. Environmental Protection Agency (US EPA) and several federal agencies created a workgroup and hosted two bed bug summits in April 2009 and February 2011, which aimed to develop a federal strategy for bed bug management (US EPA 2014).

Although there were speculations of bed bugs being a vector of several pathogens, Burton (1963) concluded that no definitive evidence linked any disease to *C. lectularius*, and thus they have not been considered as effective vectors. However, Delaunay et al. (2011) and Adelman et al. (2013) speculated that bed bugs could be involved in pathogen or arbovirus transmission to humans under special circumstances. Recently, Salazar et al. (2014) found bed bugs efficiently and bidirectionally transmitted a protozoan (*Trypanosoma cruzi* Chagas) between infected and uninfected mice in a laboratory experiment.

Since the reemergence of bed bugs, millions of dollars have been spent in controlling and reducing the spread of this pest, settling lawsuits resulting from bed bug bites in hotels, or between tenants and property owners for payments for bed bug control (Whitford 2012, Morgan 2013). According to Curl (2011), estimated revenue generated from bed bugs management in 2010 was US\$319 million.

When controlling bed bugs, pest management professionals (PMPs) have to be extremely careful about claims of elimination of populations because reinfestations are possible and could be difficult to differentiate. Therefore, it becomes very critical to identify a population based on molecular genetic techniques. Booth et al. (2011) and Saenz et al. (2012) have designed microsatellite markers to differentiate bed bug populations from the East Coast. However, no data exist on the bed bug population variation from other parts of the United States. Moreover, with the high frequency of international travel to and from the United States, the bed bugs could have been reintroduced from locations in Asia, South America, Africa, or all. Microsatellite markers could facilitate identification of these different populations and determine their origin. Once a base line for the bed bug population in an area is established, PMPs could submit specimens collected for identification, and would be able to definitively conclude which populations are genetically different, whether reinfestation was from new population, or when inadequate pest control has occurred. If genetic analysis were conducted on a regular basis, they would also be able to identify when new populations are introduced to that area.

Microsatellites are short tandem repeats of DNA sequence with 1–6 bp in length (mono-, di-, tri-, tetra-, penta-, and hexa-mers), most likely located in the noncoding regions (Korneev 1988, Tautz 1993, Goldstein et al.1995). The short tandem repeats of microsatellites are widespread in DNA and assumed to be evenly distributed over genomes (Stallings 1992, Dietrich et al. 1996, Primmer et al. 1996). Microsatellite markers have emerged as a reliable source to investigate the genetic structuring of populations to estimate the links and patterns of gene flow among populations (Vargo 2000, Balloux and Lugon-Moulin 2002, Saenz et al. 2012, Betson et al. 2014).

In the current study, eight microsatellite markers were used to measure genetic diversity and establish population structure of bed bugs collected from 10 locations in the Midwest, United States.

## **Materials and Methods**

### ***Bed Bug Collection***

During the summers of 2011 and 2012, bed bugs were collected from 95 sites from Nebraska and Missouri. The 10 collection sites included in this study were from locations where >10 adult specimens were collected (table 1). The bed bug samples for genetic analysis were selected from four cities including Lincoln, Papillion, and Omaha (Nebraska) and Kansas City, Missouri. Each sample originated from a single apartment or a single-family home in the respective cities. The samples were individually preserved in vials containing 100% ethanol and stored at –20°C until DNA extraction and genetic analysis.

**Table 1.** Location and identification of bed bug populations analyzed ( $n = 8-10$  individuals per location)

State	City	Street	Identification	Date collected	
Nebraska	Lincoln	Lincoln Mall	LNK1	June 2011	
		D Street	LNK2	Jan. 2012	
		15th Street	LNK3	June 2012	
		L Street	LNK4	May 2011	
	Omaha	Franklin Street	OMA1	June 2011	
		Boyd Street	OMA2	Aug. 2011	
		Blondo Street	OMA3	June 2011	
	Missouri	Papillion	Barrington Pkwy	PAP	July 2011
		Kansas City	Pennsylvania Ave	KAN1	May 2012
Baltimore Ave			KAN2	May 2012	

### *Genomic DNA Extraction and Genotyping*

The DNA from individual adult (male and female) bed bugs was extracted using Gentra Puregene Tissue Kit (Qiagen, Valencia, California), and a modification of their protocol. The ethanol from the samples was vaporized prior to genomic DNA extraction. The samples were homogenized in a cell lysis solution containing proteinase K at 4°C in a 1.5-ml microcentrifuge tube using a pestle. The lysate was incubated at 55°C for 3h followed by RNase treatment and protein precipitation. The DNA from the aqueous phase was precipitated using isopropanol followed by two wash with 70% ethanol. The resulting DNA pellet was air-dried, dissolved in nuclease free water, and the DNA quantified using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Inc.). The DNA concentration was diluted to between 10 and 15 ng/ml to be used in polymerase chain reaction (PCR).

Eight to 10 individuals from each of the 10 locations were genotyped using eight microsatellite markers, namely BB6B, BB15B, BB29B, BB31B, Clec37, Clec91, Clec97, and Clec104 (Booth et al. 2012). All primer sets were purchased from Invitrogen (Carlsbad, California). The forward primers were labeled with one of the three WellRED Fluorescent labels (D2, black; D3, green; and D4, blue) to assist detection on the Beckman Coulter CEQ 8000 Genetic Analyzer (Beckman Coulter Fullerton, California). Each loci was run separately. PCRs were done in 20-ml total volumes, consisting of 4 µl of 5 × PCR buffer (Phusion, New England Biolabs), 400mM dNTPs, 10–15 ng of DNA template, 0.2 µl of Phusion High-Fidelity DNA Polymerase, 0.025 µM primer concentration and RNase/DNase-free distilled water added to volume. Amplification was performed in an Applied Biosystem (Life Technologies Corporation, Carlsbad, California) 96-well thermocycler using the following conditions: initial denaturation for 30 s at 98°C followed by 25 cycles of denaturation at 98°C for 5 s annealing at 61 or 59°C for 10 s, extension at 72°C for 5 s, and the final extension at 72°C for 5min. The samples were stored at 4°C until further processing.

The amplified products were desalted following Beckman Coulter CEQ Fragment Analysis System (Beckman Coulter, Fullerton, California) protocol (P/N 608113-AF). The desalting involved precipitation of DNA using 20mg/ml glycogen (Fisher Scientific, Pittsburgh, Pennsylvania) 3M sodium acetate (Invitrogen Carlsbad, California), 95% etha-

nol, and 70% ethanol (Sigma Aldrich, St. Louis, Missouri). The precipitated DNA was re-suspended in the sample loading solution prior to separation and fragment sizing using capillary electrophoresis. The samples were loaded in 96-well plates along with fluorescent-labeled DNA standards and a layer of mineral oil to prevent evaporation. The Beckman Coulter DNA size standard kit – 400 (red) was used for a standard. The PCR was conducted in 96-well plates using Phusion polymerase following the manufacturer's instructions (New England Biolabs, Ipswich, Massachusetts). The data from the capillary electrophoresis were analyzed by the CEQ 8000 fragment analysis software version 8.0 and the allele sizes were determined from the electropherograms.

### ***Data Analysis***

Multiple software programs were used to analyze the genotyping data derived from the 10 different populations. Standard genetic distance was used to estimate genetic differentiation. The  $F_{IS}$  and  $F_{ST}$  values, the  $H_E$ – $H_o$  variation, and test for HW equilibrium were used to determine whether the populations were inbred or not. The format conversions for analyzing the data in various software programs were achieved using the CONVERT software version 1.31 (Glaubitz, 2004). Cities, states, and the overall population were used as separate groups for analysis. The genetic differentiation and inbreeding structure was determined by calculating  $F_{ST}$ ,  $F_{IS}$ , and CIs using the FSTAT version 2.9.3.2 (Goudet 1995). Relatedness was also calculated using FSTAT software using the standard genetic distance option. The 95% CI for the  $F_{ST}$  values were estimated by bootstrapping over loci and the  $P$ -values were obtained through multiple permutations. The pairwise genotypic differentiation was derived using the G-test (Gaudet 1996) found in the web version of GENEPOP (Raymond and Rousset 1995). The exact tests for linkage disequilibrium and deviation from Hardy-Weinberg equilibrium were also conducted using the web version of GENEPOP. The expected and observed heterozygosity for the populations were calculated with the aid of the Genetic Data Analysis Software (version 1.1, Lewis and Zaykin 2001). A two-tailed  $t$ -test (Saenz et al. 2012) provided the  $P$ -value to determine the significance of the heterozygosity analysis. The genetic distance between the various populations was determined using a neighbor-joining unrooted tree (1,000 iterations of bootstrap) using the POPTREEW software (Takezaki 2014). ARLEQUIN software version 3.01 (Excoffier et al. 2005) provided the number of alleles per loci and the total number of alleles per population.

## **Results**

### ***Bed Bug Genetic Diversity***

The electropherograms obtained through capillary electrophoresis of the PCR products showed distinct peaks of alleles for the various loci analyzed. Alleles in a single sample were either of the same size as single peak (homozygous) or two sizes as two peaks (heterozygous). Reruns were made for specimens where PCR or CEQ detection failed. All eight loci were polymorphic, with a total of 8 to 16 alleles per locus and a maximum of 6 alleles at one locus in a single population (table 2). The mean number of alleles ranged from 1.63 to 3.38 across the populations tested. The expected and observed heterozygosity over all

loci was 0.883 and 0.406, respectively. The expected heterozygosity differed significantly from the observed heterozygosity for all loci tested ( $t$ -value = 2.45;  $df = 6$ ;  $P = 0.0001$ ; Fisher's exact test  $\chi^2 = \infty$ ;  $df = 124$ ;  $P < 0.0001$ ; table 3).

**Table 2.** The number of alleles at each locus related to each bed bug population

Locus ID	Total No.	Populations										Mean	S.D.
		LNK 1	LNK 2	LNK 3	LNK 4	OMA 1	OMA 2	OMA 3	PAP	KAN 1	KAN 2		
BB6B	11	1	2	5	1	3	2	3	3	1	5	2.6	1.5
BB15B	16	2	5	4	2	4	3	4	4	2	3	3.3	1.1
BB29B	16	2	4	6	4	3	4	2	4	2	3	3.4	1.2
BB31B	8	2	4	4	2	1	4	2	3	3	3	2.8	1.0
Clec37	11	2	2	1	1	5	4	3	2	1	2	2.3	0.8
Clec91	12	5	4	2	1	3	2	2	3	1	2	2.5	1.3
Clec97	12	4	3	3	1	5	5	3	4	3	3	3.4	1.2
Clec104	10	3	3	3	1	3	2	2	2	1	3	2.3	1.3
Mean	12	2.63	3.38	3.50	1.63	3.38	3.25	2.63	3.13	1.75	3.00	2.83	
S.D.	2.78	1.3	1.1	1.6	1.1	1.3	1.2	0.74	0.74	0.89	0.73	1.09	

The mean and total number of alleles per locus are also included ( $n = 8-10$  individuals per location).

**Table 3.** A comparison of observed ( $H_o$ ) and expected heterozygosity ( $H_e$ ) of microsatellite loci ( $n = 8-10$  individuals per location)

Locus ID	$H_o$	$H_e$
BB15B	0.863	0.880
BB29B	0.633	0.883
BB31B	0.438	0.728
BB6B	0.113	0.839
Clec104	0.163	0.521
Clec37	0.238	0.840
Clec91	0.163	0.864
Clec97	0.638	0.850
Mean	0.406	0.883

The exact test for linkage disequilibrium resulted in no statistical significance confirming that the markers are unlinked and segregated independently. The  $F_{ST}$  values were  $>0.25$  for all the populations, which represents a high level of genetic differentiation between these populations (table 4). Because the CI of the  $F_{ST}$  did not include 0, which would represent all the populations having the same allele frequencies, the estimate was significantly different. The rest of the populations had low positive  $F_{IS}$  values, and their CI values were statistically insignificant. The relatedness between each of the populations was  $>0.5$ , indicative of sibling or parent-offspring relationships. The CI did not include 0 and represented a significant difference. The G-test for all pairs of population showed high significance, which denotes an extremely high level of genetic differentiation. The  $P$ -values estimated by permutations for pairwise tests of differentiation also appeared to be of high statistical

significance ( $P = 0.002$ ), except for the Kansas City population that showed moderate significance ( $P = 0.048$ ; table 5).

**Table 4.**  $F_{ST}$  and G-test results of  $F$ -statistics analysis, grouped as cities, state, and overall population ( $n = 8-10$  individuals per location,  $N =$  number of locations)

	Region	$N$	$F_{ST}$ (95% CI)	G-test
Within city	Kansas city (KAN)	2	0.572 (0.42–0.697)	<0.002
	Omaha (OMA, PAP)	4	0.404 (0.29–0.509)	<0.002
	Lincoln (LNK)	4	0.522 (0.41–0.63)	<0.002
Within state	Nebraska	8	0.456 (0.369–0.541)	<0.002
Total population	Midwest	10	0.481 (0.397–0.57)	<0.002

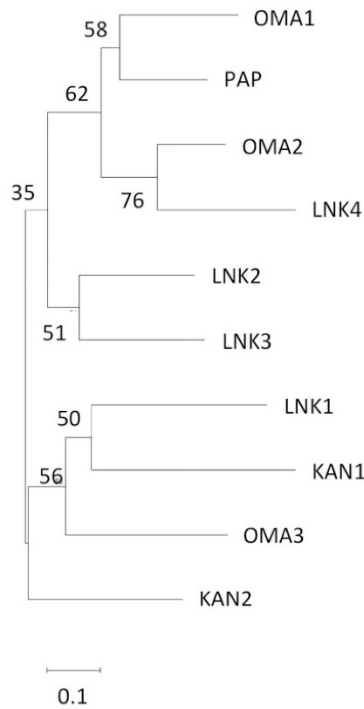
**Table 5.**  $F_{IT}$ ,  $F_{IS}$ , and  $r$  analysis, 95% CI, and G-test results grouped as cities, state, and overall population ( $n = 8-10$  individuals per location)

Populations	Region	$F_{IT}$ (CI)	$F_{IS}$ (CI)	$r$ (CI)	P value*	G-test
Within City	Kansas city (KAN)	0.485 (0.205–0.739)	–0.218 (–0.498–0.196)	0.771 (0.663–0.845)	0.048*	Significant
	Omaha (OMA, PAP)	0.543 (0.318–0.76)	0.228 (–0.039–0.575)	0.525 (0.396–0.625)	0.017**	Highly significant
	Lincoln (LNK)	0.543 (0.347–0.736)	0.033 (–0.159–0.321)	0.679 (0.59–0.748)	0.008**	Highly significant
Within state	Nebraska	0.524 (0.325–0.722)	0.117 (–0.095–0.433)	0.599 (0.53–0.66)	0.002**	Highly significant
Total Population	Midwest	0.516 (0.312–0.718)	0.057 (–0.161–0.375)	0.636 (0.575–0.689)	0.002**	Highly significant

P-value, \* = significant at  $P < 0.05$ , \*\* $P < 0.02$

The genetic distance between each population was estimated using a neighbor-joining distance tree (fig. 1). There were three clusters: four populations from Omaha and Lincoln; two Lincoln, Nebraska, populations; and two Missouri populations that were grouped in the same cluster with two populations from Nebraska (Lincoln and Omaha). The populations were not separated by geographic location.





**Figure 1.** A neighbor-joining distance tree depicting the genetic distance of the overall bed bug populations. The bootstrap values and a scale for the distance are also provided below the tree. Lincoln (LNK), Omaha (OMA), Kansas City (KAN), and Papillion (PAP).

## Discussion

The resurgence of the bed bugs has been speculated to be a cause of increased travel, greater resistance to insecticides, lack of social awareness, and incomplete pest control (Pinto et al. 2007, Reinhardt and Siva-Jothy 2007, Doggett et al. 2011). Previous studies by Booth et al. (2012) and Saenz et al. (2012) on the genetic diversity of bed bugs in the eastern United States demonstrated that microsatellite markers were a useful tool to differentiate bed bug populations within an apartment building or even across large areas (Massachusetts to Florida). Our report is the first analysis of bed bug populations in the Midwest, including Nebraska and Missouri. According to Orkin Pest Control in 2012, Omaha, Lincoln, Kearney, and Hastings, Nebraska, were among the top bed bug-infested cities in America (Peters 2013). Omaha moved from No. 28 to No. 17 on their list of the top 50 bed bug-plagued cities. The list is based on the amount of treatments they conducted in those areas. The fact that all these cities have University campuses that generate immense international and domestic travel is significant, especially as humans play an important role in the passive dispersal of bed bugs.

Of the eight microsatellite markers analyzed, six of them (bb6b, bb15b, bb29b, bb31b, clec91, and clec97) had more than four alleles showing high heterozygosity, thereby differing from the previous reports in eastern United States (Saenz et al. 2012). All loci used in

this study had unique alleles in addition to those previously identified by Saenz et al. (2012) and Booth et al. (2012). The possible explanation that fits this scenario is outbreeding and random mating by the female bed bugs. Another reason could be more than one male's sperm was used to fertilize the female bed bug's eggs. Additionally, with the increase in the infestation level, there would be an increase of possible mates and mating with several males could occur.

Four of the 10 populations (one each from Lincoln, Omaha, Papillion, and Kansas City) showed more than four alleles per loci, suggesting that probably the population was formed when a single female mated a single male with the same allelic composition or a different allelic composition. Another possible reason could be the infestation level at these sites was low, thus reducing the number of adult males available to add to the gene pool. The rest of the populations showed heterozygote advantage maybe because of random mating or multiple sources introduced at one time, allowing for the exchange of multiple alleles. This agrees with studies by Szalanski et al. (2008) and Booth et al. (2012). The difference in these two studies is the geographic area they encompass. The Szalanski et al. (2008) study covers a wider region and includes the southern Midwest, whereas the Booth et al. (2012) study was limited to one apartment building.

Booth et al. (2012) suggested that a combination of genetic diversity and pyrethroid resistance could provide evidence for the possible sources of heterozygosity. Pyrethroid-resistant bed bugs have been documented globally (Davies et al. 2012) in Europe (Durand et al. 2012, Kilpinen et al. 2011), Asia (Tawatsin et al. 2011, Swannayod et al. 2010), and Australia (Lilly et al. 2010). The  $F_{ST}$  values of  $>0.25$  for all the populations suggested high genetic differentiation, which was also statistically significant at the 95% CIs.

The genetic distance for all the populations using a neighbor-joining tree showed that the populations from Kansas City were associated genetically with populations from Lincoln and Omaha. The relatedness coefficient ( $r$ ) for the Kansas City populations was 0.771, suggesting a high level of relatedness, possibly because of the apparent lack of gene flow, due to the limited mobility of bed bugs in an aggregation. The population from Papillion was clustered together with a population from Omaha, which could be possible because the distance between the two cities was  $<16$ km. The bed bug populations from the Midwest did not form clusters specific to the geographic locations from where the samples were collected.

In conclusion, the results from this study reinforce the effectiveness of microsatellite markers in identifying the relatedness of bed bug populations collected from different locations. It is a fast and reliable method. Once a base line for the bed bug population in an area is established, PMPs could submit specimens collected for identification and would be able to definitively conclude, when populations are different, whether reinfestation was from a new population or when pest control was inadequate. If genetic analysis were conducted on a regular basis, they also would be able to identify when new populations are introduced to an area because there were highly significant differences between and within the cities. Of the 10 locations tested, bed bug populations formed three clusters. The populations appeared to depart from Hardy-Weinberg equilibrium, which was owing to high heterozygosity. Based on the allelic variation, no definitive conclusion could be made on

how these populations were established; it could be from single or multiple sources/infestations. Several new alleles were identified for five of the eight microsatellites used in this study. The new alleles increased the range of the five microsatellites (table 6). For the remaining three microsatellites, the alleles identified in this study were all within the range identified by Booth et al. (2012).

**Table 6.** Allele size range for bed bug microsatellites

Locus ID	Alleles size range		
	Booth et al. 2012	Additional	Final
BB6B	137–163	125–142	125–163
BB15B	129–187	*	129–187
BB29B	178–268	*	178–268
BB31B	196–218	174–192	174–218
Clec37	208–268	180–248	180–268
Clec91	157–184	139–170	139–187
Clec97	295–316	236–298	236–316
Clec104	236–266	*	236–266

\*No new additional alleles

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