Journal of Pest Science (2026) 99:121
https://doi.org/10.1007/s10340-026-02089-z

Admixture without recurrent intercontinental gene flow: contrasting patterns of genetic diversity and knockdown resistance profiles reveal independent evolution in resurgent common bed bug populations

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Received: 2 March 2026 / Revised: 4 August 2026 / Accepted: 5 August 2026

Abstract
The common bed bug (*Cimex lectularius*) was largely eliminated from industrialized countries by mid-twentieth century insecticide campaigns including the widespread application of DDT. However, by the late 1990s, populations were resurging on a global scale. This rapid reemergence has been widely attributed to increased rates of international travel facilitating frequent intercontinental dispersal of insecticide-resistant populations. Because bed bugs disperse almost exclusively through human-mediated means, this scenario predicts that frequent gene flow should homogenize populations globally and enable the rapid worldwide spread of resistance alleles. We tested this prevailing hypothesis using microsatellite markers and knockdown resistance (*kdr*) genotyping from populations across the United States, Canada, and Europe, sampled within approximately ten years of resurgence onset. Contrary to expectations, we found strong regional genetic structure indicating limited intercontinental gene flow. U.S. populations exhibited significantly higher within-population allelic diversity than European populations, likely through admixture of historical U.S. lineages, while Europe maintained the highest regional allelic richness. Furthermore, *kdr* profiles differed dramatically. Specifically, while the L925I mutation is ubiquitous across regions, the V419L resistance-associated mutation was present in over 55% of USA populations (in combination with the L925I mutation) but virtually absent from Europe and Canada. These findings challenge the prevailing narrative, suggesting the resurgence reflects regional expansion of evolutionarily independent lineages that persisted in cryptic refugia rather than frequent intercontinental mixing during early resurgence, and have important implications for predicting resistance spread.

Keywords *Cimex lectularius* · Population structure · Invasion genetics · Urban pest · Human-mediated dispersal · Cryptic refugia

Introduction

The common bed bug (*Cimex lectularius*) provides a striking example of pest resurgence following apparent near eradication. Likely associated with human societies since the dawn of civilization (Miles et al. 2025a), this species was a ubiquitous human ectoparasite prior to World War II (Ebling 1975; Usinger 1966; Potter 2018). Following the introduction of DDT for indoor pest control, bed bug populations declined dramatically across industrialized nations. While populations of bed bugs resistant to DDT were reported

Subject Editor Maohua Chen.

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Published online: 09 September 2026

shortly after its widespread application (Johnson and Hill 1948; Busvine 1958), emerging populations were likely suppressed by periodic introductions of new broad-spectrum insecticides throughout the mid-to-late twentieth century (Usinger 1966; Busvine 1980). For nearly five decades, bed bugs remained rare in developed countries, with infestations considered uncommon and of minimal health concern (Potter 2018). In fact, by the 1990s, few pest controllers had ever encountered an infestation (Pinto et al. 2007). However, beginning in the late 1990s, a rapid and near-parallel resurgence of bed bugs occurred across North America, Europe, Australia, and parts of Asia, reestablishing themselves as a major urban pest (Doggett et al. 2012; Potter et al. 2008). This resurgence has imposed substantial economic costs and generated significant public health concerns due to psychological distress, cutaneous reactions, allergic response, and insecticide exposure during control efforts (Doggett et al. 2012; Price et al. 2012; Gordon and DeVries 2024).

The near-simultaneous global resurgence has been widely attributed to the combined effect of two factors: increased international travel facilitating intercontinental dispersal, and the evolution of insecticide resistance enabling population establishment and persistence (Reinhardt and Siva-Jothy 2007; Romero et al. 2007). Because bed bugs lack active dispersal abilities outside of contiguous structures and move almost exclusively through passive transport on human belongings including furniture and luggage (Pinto et al. 2007; Doggett and Russell 2009), their contemporary distribution is fundamentally shaped by the movement of humans (Doggett et al. 2004; Potter et al. 2008; Zorrilla-Vaca et al. 2015; Lewis 2024). As such, the exponential increase in international air travel since the 1990s (ICAO (International Civil Aviation Organization), 2018) has frequently been cited as a major driver of the species' global resurgence (Paul and Bates 2000; Boase 2001; Reinhardt and Siva-Jothy 2007; Ter Poorten and Prose 2005; Doggett et al. 2012). Under this scenario, the increase in opportunities for long-distance dispersal, including frequent intercontinental gene flow, should homogenize genetic variation globally creating admixed cosmopolitan populations lacking strong geographic structure. Paradoxically, while the resurgence is attributed to increased travel, variation in *kdr* profiles suggests that intercontinental gene flow may be more limited than assumed, with dispersal occurring primarily within, rather than between, continents (Zhu et al. 2010; Durand et al. 2012; Booth et al. 2015; Dang et al. 2015; Palenchar et al. 2015; Balvín and Booth 2018; Holleman et al. 2019; Cho et al. 2020; Lewis et al. 2023; Booth 2024; Castex et al. 2025; Yu et al. 2026).

An alternative, largely unexplored hypothesis is that contemporary infestations might represent expansions from cryptic refugial populations that persisted through the insecticide era rather than from recent reintroductions from distant sources (Szalanski et al. 2008). Under this 'cryptic refugia' scenario, small populations would have survived in areas where insecticide exposure or efficient pest control was limited (e.g., low-income housing in rural communities, poultry farms), maintaining the genetic legacies of historical colonization that predated the widespread application of DDT. Bed bugs likely spread across Americas from their Old-World origin with early European explorers, who introduced them first into seaports followed by inland dispersal (Marlatt 1916; Usinger 1966; Ebling 1975; Potter 2018; Naylor et al. 2018a). The subsequent 1990s resurgence would then reflect regional expansion from cryptic refugia following the evolution or selection of insecticide resistance. Resistance mechanisms in bed bugs are diverse (Dang et al. 2017), but particular attention has been focused on *kdr*-associated mutations in the voltage-gated sodium channel that confer resistance to commonly used pyrethroids (Yoon et al. 2008; Dang et al. 2015). This cryptic refugia scenario predicts that early in the resurgence era bed bug populations would be characterized by: 1) strong regional genetic structure reflecting historical introduction patterns, 2) region-specific resistance profiles indicating independent evolutionary trajectories, and 3) elevated within-population diversity where multiple historical lineages co-persisted in cryptic refugia and subsequently admixed during expansion, a pattern sometimes described as 'genetic ghosts' where the genetic signature of extinct or unsampled populations are left detectable in living or later populations revealed through admixture signals (Willerslev and Meltzer 2021). Distinguishing between the competing hypotheses of contemporary global mixing versus expansion from cryptic refugia has fundamental implications for understanding the evolutionary dynamics of resurgent pests and predicting the geographic spread of adaptive traits.

Resolving whether bed bug populations are connected by recurrent gene flow or evolving independently has critical practical implications for pest management. If intercontinental dispersal homogenizes populations, resistance alleles arising in one region could spread worldwide, necessitating globally coordinated management strategies and suggesting limited time windows for regional interventions before resistant genotypes become cosmopolitan. Conversely, if populations are evolutionarily independent, regional resistance profiles should remain distinct over management-relevant timescales, allowing geographically tailored control strategies adapted to regional resistance profiles, as demonstrated in other systems where resistance varies spatially (e.g., *Anopheles* mosquitos) (Protopopoff et al. 2008; ffrench-Constant 2013; Hemingway et al. 2016). While insecticide resistance can spread rapidly within regions when gene flow is high, as documented in mosquitos (Lynd et al. 2010) and more recently suggested by *kdr* distribution patterns in bed bugs (Lewis et al. 2023; Booth 2024),

the rate and geographic extent of intercontinental resistance spread in bed bugs remains empirically untested.

Here, we combine microsatellite markers and *kdr*-associated mutations to genotype *C. lectularius* populations from the United States, Canada, and Europe collected within the first decade of the global resurgence (2009–2011), testing competing hypotheses about population connectivity and evolutionary independence. The combination of both neutral markers (microsatellites) that reflect demographic history and functional loci under strong selection (*kdr*) that reveal adaptive evolution enables robust inference about both historical connectivity and contemporary adaptive evolution. We specifically addressed three questions to distinguish between the global homogenization hypothesis and the cryptic refugial hypothesis: 1) do patterns of neutral genetic diversity support frequent intercontinental gene flow during the early stages of the bed bug resurgence, or historical isolation followed by regional expansion; 2) how is genetic diversity partitioned within vs. among populations at regional scales; and 3) are *kdr*-associated allele frequencies homogeneous across regions, as expected under frequent gene flow, or geographically structured indicating independent evolution? Our findings challenge the prevailing narrative of homogenization through intercontinental travel and instead support evolutionary independence of regional populations. These findings have important implications for understanding the spread of resistance-associated alleles and tailoring region-specific management strategies.

Materials and methods

Sample collection, DNA extraction and microsatellite genotyping

Bed bug samples were collected from human dwellings between 01 December 2009 and 09 January 2011 by regional pest management professionals. These collections occurred roughly ten years after the onset of the species' resurgence (Davies et al. 2012). In total, 971 individual bed bugs were obtained from 109 populations across nine European countries ($n = 65$ populations), three Canadian provinces ($n = 9$), and 17 U.S. states ($n = 35$) (Fig. 1a; Supp. Table 1). Specimens were preserved in 96% ethanol immediately after

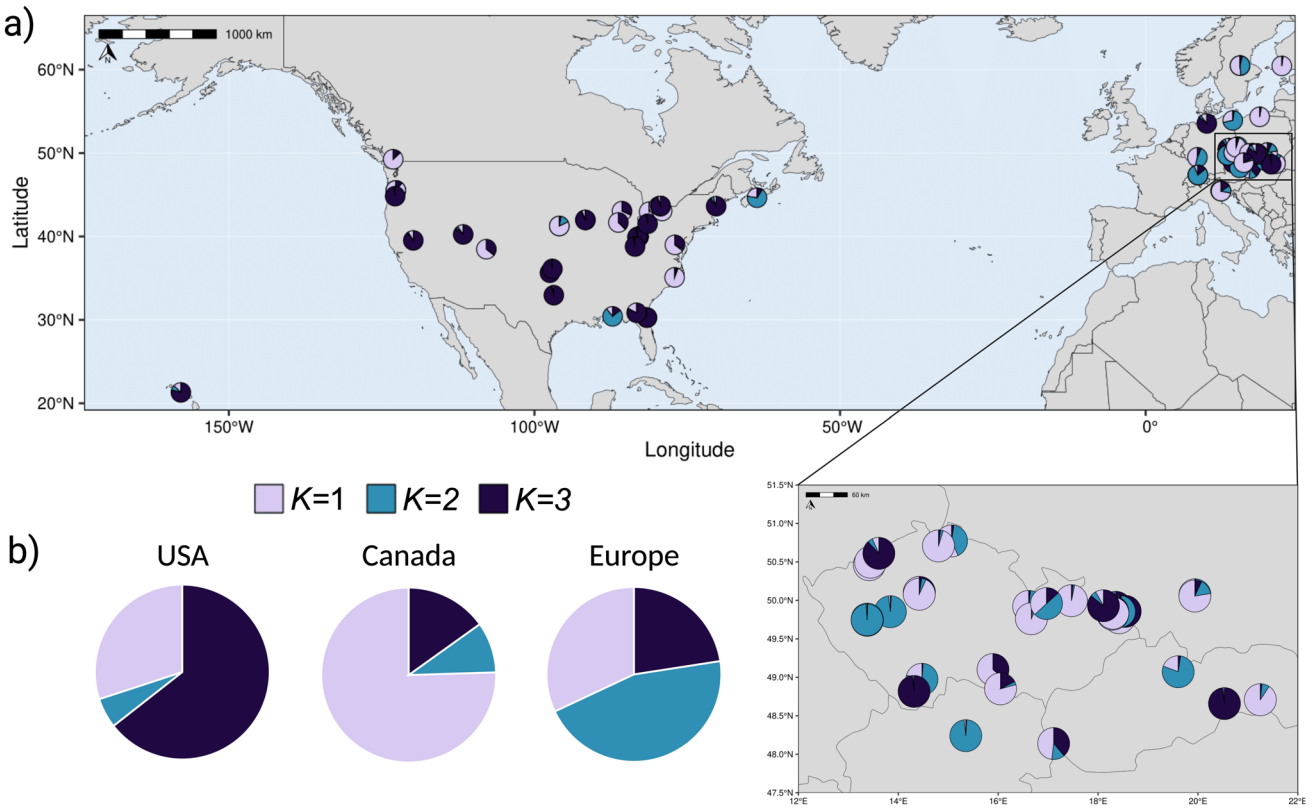

Fig. 1 Population genetic structure of *Cimex lectularius* across North America and Europe. **a** Geographic distribution of STRUCTURE assignment probabilities for individual bed bugs collected from the United States, Canada, and Europe. Each pie chart represents a sampling location, with colors indicating proportional membership in three genetic clusters (*K*1–*K*3) inferred at $K = 3$. The inset highlights fine-scale spatial variation among European sampling locations. **b** Mean cluster membership proportions for samples from the United States ($n = 35$), Canada ($n = 9$), and Europe ($n = 65$)

collection and stored at − 18 °C until processing. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) and stored at − 18 °C until analysis. Individuals were genotyped at 14 polymorphic microsatellite loci: *BB15B, BB31B, BB38B, BB42B, Clec6, Clec11, Clec37, Clec45, Clec48, Clec90, Clec96, Clec97, Clec99, and Clec104* (Booth et al. 2012). PCR amplification and fragment analysis were conducted on a Li-Cor 4300 dual-laser DNA analyzer (Lincoln, NE, USA) following the protocols described in Booth et al. (2012).

Population genetic analyses

All statistical analyses were conducted in R v4.3.0 (www.R-project.org). Despite an average sample size of 8.9 individuals per population, sample sizes ranged from 1 to 11. To evaluate the effect of this variation, allele-frequency estimates from subsampled datasets of 3 individuals per population (10 replicates of randomly selected individuals) were compared with estimates based on 10 individuals per population for four randomly chosen populations. For each population, both Pearson correlation coefficients and root mean square errors were calculated (Supp. Figure 1). Across most populations subsample estimates closely mirrored full-sample frequencies, with high Pearson correlation (≥ 0.9) and low root mean square error values, indicating that small samples can effectively capture population-level genetic variation. Note that only four populations exhibited sample sizes of three or less, with the modal value being ten. As such, all populations were included in subsequent analyses. Summary statistics, including number of alleles and observed and expected heterozygosity, were calculated in the *poppr* package (Kamvar et al. 2014, 2015). Allelic richness was estimated with the *vegan* package (Oksanen et al. 2025). The mean within-population number of alleles was calculated using the *adegenet* package (Jombart 2008). To test for differences among geographic regions (USA, Canada, and Europe), a Welch's one-way ANOVA was conducted, which does not assume equal variances across groups. Post-hoc pairwise comparisons were performed using pairwise t tests with Bonferroni correction and non-pooled standard deviations to identify which regions differed significantly. Weir and Cockerham (1984) estimates of pairwise F_{ST} were calculated using the hierfstat package (Goudet 2005).

Population structure was evaluated using multiple complementary approaches with differing assumptions. First, Bayesian clustering was performed in STRUCTURE v2.3.4 (Pritchard et al. 2000) using a burn-in of 50,000 iterations followed by 250,000 MCMC iterations, with 10 replicate runs for each value of K (2–5). To minimize bias associated with uneven sampling and the inclusion of highly related individuals (see VonHoldt et al. 2010; Booth et al. 2015), random subsets consisting of one individual per population were generated for each run following Booth et al. (2015) using a custom R script. Output files were processed with StructureHarvester (Earl & vonHoldt 2012) to estimate the most likely K using the ΔK method of Evanno et al. (2005), and replicate runs were aligned using CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007). STRUCTURE results were visualized in R using *ggplot2* (Wickham 2016). Because Bayesian clustering methods provide simplified summaries of genetic variation rather than discrete biological populations, results were interpreted cautiously and in conjunction with additional analyses. As such, STRUCTURE clusters should be interpreted as statistical groupings that summarize shared genetic signal rather than discrete evolutionary lineages or true biological populations. Second, multivariate clustering was assessed using discriminant analysis of principal components (DAPC) implemented in *adegenet*, with both state/country- and region-level (Europe, Canada, USA) groupings used as prior strata. Finally, spatial genetic structure was further explored using spatial principal component analysis (sPCA), also implemented in *adegenet*, with results visualized in R. As multivariate analyses such as DAPC and sPCA are robust to unequal sample sizes, all individuals were included.

Knockdown resistance profiling

Knockdown resistance profiles were determined for 98 populations (USA = 33, Canada = 9, Europe = 56; Supp. Table 1) following Booth et al. (2015). From each population, two fragments of the voltage-gated sodium channel gene were amplified in three randomly chosen individuals, targeting the V419L and L925I mutations associated with pyrethroid resistance (Zhu et al. 2010). While it would be ideal to sequence all individuals sampled for each infestation, previous studies have provided justification that a single to few individuals per population are representative of the population genotypic frequency (see Booth et al. 2015; Balvin and Booth 2018; Holleman et al. 2019; Block et al. 2025). Purified PCR products were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM® 3100-Avant Genetic Analyzer or by a commercial sequencing service (Macrogen Inc.). Resulting chromatograms were aligned and scored at the resistance-associated loci: amino acid position 419 (wild type GTC = valine; mutant CTC = leucine) and position 925 (wild type CTT = leucine; mutant ATT = isoleucine). Heterozygous genotypes were identified by the presence of overlapping nucleotide peaks at the target sites. Within-population genotypes were combined to generate population-level *kdr* profiles following Lewis et al. (2023).

Results

Population genetic analyses

DNA was extracted from a total of 971 individuals with between 1 and 11 specimens per populations (mean—Overall 8.9, USA 8.65, Canada 9.2, Europe 9.56). Pooling of DNA per population was not performed. Across loci, allelic diversity was moderate to high, ranging from 2 (*Clec6*, *Clec48*) to 27 alleles (*BB15B*), with a mean of 10.07 alleles per locus (Table 1). Expected heterozygosity was, in general, moderate to high (0.38–0.83), with one outlier (*Clec48*, 0.11) (Table 1). This outlier also exhibited the fewest alleles per locus. In contrast, observed heterozygosity was uniformly low, ranging from 0.05 to 0.27 (Table 1). When considered globally, all loci exhibited significant departures from Hardy Weinberg Equilibrium following Bonferroni correction ($p = \leq 0.001$). Summary statistics are presented in Table 1. Considered regionally (USA, Canada, Europe), observed heterozygosity differed among the three populations. The U.S. population exhibited the highest mean heterozygosity (0.231 ± 0.116), followed by Canada (0.161 ± 0.127) and Europe (0.124 ± 0.063). A mixed-effects model accounting for variation among loci and weighting each H_O estimate by the number of individuals in each population revealed a significant effect of population ($F_{2, 26} = 5.58$, $p = 0.0096$). Post-hoc pairwise comparisons using Welch-corrected t tests with Bonferroni correction indicated that H_O in the United States was significantly higher than in Europe ($p = 0.02$), whereas differences between the United States and Canada ($p = 0.43$) and between Canada and Europe ($p = 1.00$) were not statistically significant. The smaller sample size from Canada likely reduced the power to detect differences in H_O involving this population in comparisons. Significant population differentiation was detected across all pairwise F_{ST} comparisons ($P = 0.0009$) (USA—Europe: 0.080; USA—Canada: 0.097; Canada—Europe: 0.104).

Table 1 Global summary statistics for bed bug, *Cimex lectularius*, samples screened for 14 microsatellite loci

Locus	No. alleles	Observed heterozygosity (H_O)	Expected heterozygosity (H_E)	Allelic richness (A_r)
BB15B	27	0.20	0.83	12.20
BB31B	18	0.23	0.76	9.33
BB38B	7	0.19	0.69	5.05
BB42	16	0.24	0.83	9.29
Clec6	2	0.06	0.50	2
Clec11	4	0.23	0.56	3.16
Clec37	6	0.17	0.58	4.14
Clec45	3	0.09	0.53	2.33
Clec48	2	0.06	0.11	1.99
Clec90	9	0.27	0.78	5.00
Clec96	8	0.07	0.50	4.12
Clec97	15	0.17	0.74	7.28
Clec99	14	0.25	0.83	7.34
Clec104	10	0.05	0.38	4.66
Mean	10.07	0.16	0.62	5.56
USA	5.9	0.23	0.59	5.95
Canada	3.4	0.16	0.45	3.42
Europe	7.3	0.12	0.59	7.31

This pattern was also observed when comparing the within-population number of alleles among regions. A Welch's one-way ANOVA revealed significant differences in the mean number of alleles per population among the three geographic regions (Welch's $F_{2, 24.67} = 5.64$, $p = 0.010$). Post-hoc pairwise comparisons indicated that U.S. populations harbored significantly more alleles per population compared to European populations ($p = 0.009$), with no significant difference between the U.S. and Canada ($p = 0.052$) or between Canada and Europe ($p = 1.00$). However, when comparing allelic richness at the regional scale (standardized across all populations within each region), Europe exhibited the highest diversity (AR = 7.31), followed by the USA (AR = 5.95) and Canada (AR = 3.42).

STRUCTURE analysis identified three distinct genetic clusters (*K*1, *K*2, *K*3) across sampling locations spanning U.S., Canada, and Europe (Fig. 1a, 2). The geographic distribution of *K* clusters revealed pronounced continental structuring, with *K*3 predominating across the U.S. (~ 70%), with minor contributions from *K*1 (~ 20%) and *K*2 (~ 10%), *K*2 dominant in Europe representing approximately 60% of ancestry with *K*1 ~ 35%, and minimal *K*3 (~ 5%), and *K*1 most prevalent in Canada (~ 70%) with subequal contributions from *K*2 and *K*3 (Fig. 1b). Within-region admixture was minimal, with most individuals showing strong assignment to a single cluster (Fig. 2). At $K = 4$ and $K = 5$, additional fine-scale structure emerged within continental groups, though the three-cluster pattern remained the most biologically interpretable organization. It should be noted that programs including STRUCTURE create simplified models of genetic variation that do not always reflect real biological populations. Instead, these models are tools that help summarize complex patterns in the data. As noted by Jombart et al. (2010) and Meirmans (2015), no single 'true' *K* exists. Instead, the different values of *K* simply provide useful summaries of the data. As such, determining the optimal number of genetic clusters using methods like that of Evanno et al. (2005) should be viewed as guides rather than final answers, and their results should be interpreted together with ecological context, geography, and other analyses (e.g., DAPC, sPCA, PCA). These clusters represent inferred patterns of shared ancestry rather than distinct evolutionary lineages, and therefore should be viewed as model-based

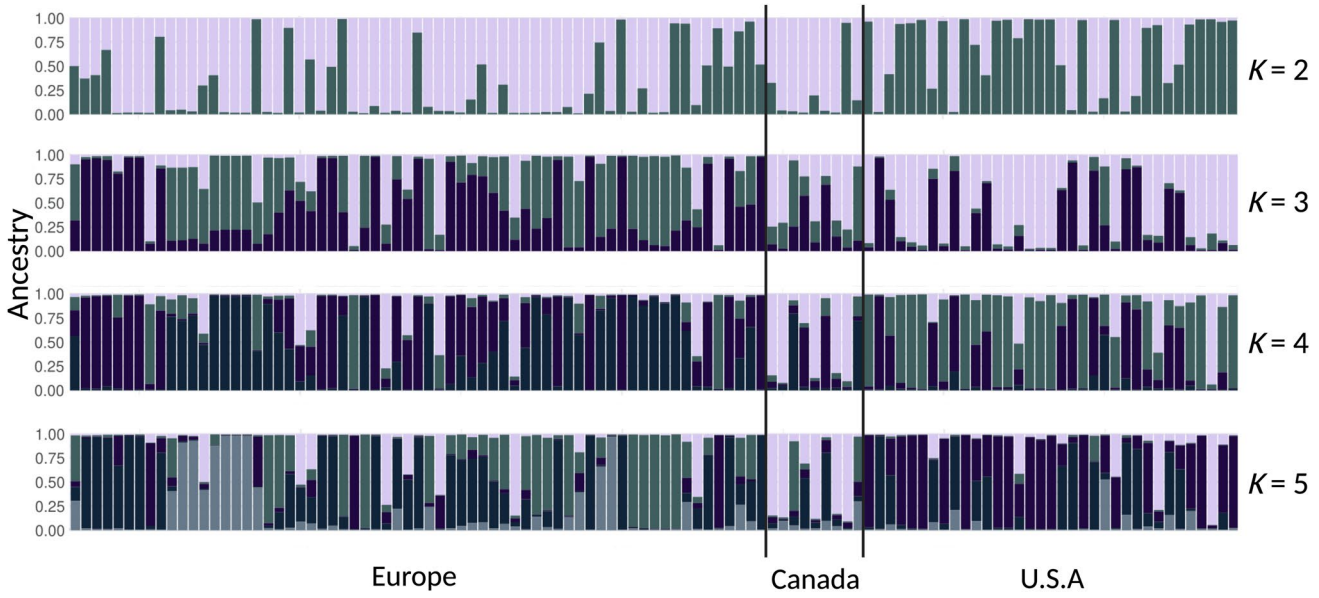

Fig. 2 STRUCTURE barplot of *Cimex lectularius* across North America and Europe. STRUCTURE assignment probabilities for individual bed bugs collected from the United States ($n=35$), Canada ($n=9$), and Europe ($n=65$). Each bar represented the proportion of cluster (K) identity explained for each individual/population

summaries of genetic structure rather than literal population units.

Discriminant Analysis of Principal Components (DAPC) based on regional groupings revealed three well-separated genetic clusters (Fig. 3). European samples formed a distinct, cohesive cluster, characterized by tight clustering and minimal dispersion. U.S. samples form a dense cluster with extensive overlap among individuals, indicating genetic homogeneity within this region. Canadian samples showed intermediate positioning between European and U.S. clusters but maintained distinct separation from both. When populations were identified by state (U.S.), province (Canada) or country (Europe), this analysis revealed complex patterns of genetic connectivity and isolation (Supp. Figure 2). European countries formed a tight, interconnected network, with Austria, Czech Republic, Switzerland, Poland, Sweden, and Italy showing overlapping distributions and numerous connections. Nova Scotia and Ontario emerged as genetically intermediate populations, positioned centrally with connections extending to both European countries and U.S. locations. U.S. states displayed more dispersed distributions, with some states (Utah, Oklahoma, Oregon, Georgia, Nevada) showing distinct positioning, while others (Ohio, Pennsylvania, Michigan, Maryland, North Carolina) clustered more centrally. Overall, the network structure demonstrated strong continental differentiation while revealing specific regions serving as genetic bridges between major population clusters. Spatial Principal Component Analysis (sPCA) demonstrated a strong latitudinal cline in genetic variation (Fig. 4). The sPCA, which considers spatial data, indicates that the European samples are genetically isolated from the U.S. and to some extent, the Canadian samples.

Knockdown resistance profiling

Genotyping of *kdr*-associated mutations across 98 populations revealed substantial geographic variation in resistance allele composition (Table 2). Wild-type (susceptible) genotypes were rare across all regions, detected in only 3.03% of USA populations, 3.57% of European populations, and completely absent in Canadian samples. The single L925I mutation showed high prevalence across all three regions but with notable frequency differences: 100% in Canada, 85.71% in Europe, and 39.39% in USA. The compound genotype L925I & V419L, carrying both resistance mutations in homozygous form, exhibited strong regional differentiation. This genotype was predominant in the USA, present in 48.48% of populations. In stark contrast, the double-mutant genotype was nearly absent from Europe (1.79%) and completely absent from Canada. The V419L mutation in isolation from L925I was not detected in any population across all regions.

Discussion

The late 1990s to early 2000s saw the simultaneous reemergence of *C. lectularius* populations across Europe and the United States (Miller 2018; Naylor et al. 2018a). Initially centered around the hospitality industry, infestations rapidly expanded into private residences (Doggett et al. 2018).

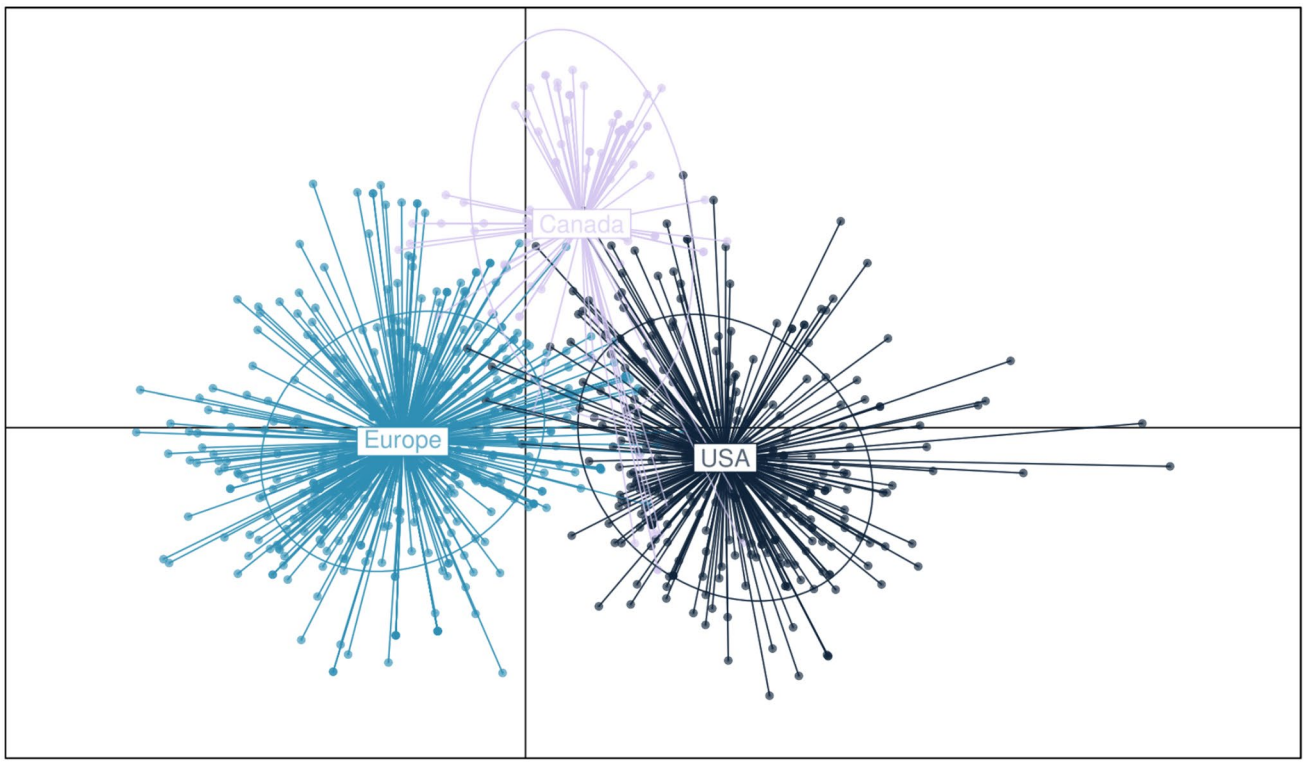

Fig. 3 Discriminant Analysis of Principal Components (DAPC) showing genetic clustering of bed bug populations from the United States ($n=35$), Canada ($n=9$), and Europe ($n=65$). Points represent individual samples projected onto the first two discriminant functions, with lines connecting individuals to their regional centroid. Ellipses indicate the dispersion of each geographic group. The compact clustering of North American samples reflects strong founder effects and genetic homogenization, whereas the broader dispersion of European samples is consistent with greater standing genetic variation and secondary contact among lineages

By 2006, bed bugs had been reported in all 50 U.S. states (Gangloff-Kaufmann et al. 2006). Although bed bugs were present across Europe at varying levels (Naylor et al. 2018a), the formation of the European Union and increased cross-border mobility likely facilitated regional spread. The late-20th-century political reorganization of Europe following the dissolution of the Iron Curtain may also have increased east–west human movement, creating renewed opportunities for contact among previously structured bed bug lineages. Thus, globalization, increased inter- and intra-continental travel, reduced insecticide efficacy associated with resistance evolution, the limited insecticidal classes available due to EU regulation, and high-density living conditions (Doggett et al. 2018) created opportunities for global spread of bed bugs. These factors are commonly invoked to explain the near-synchronous resurgence across North America and Europe (Paul and Bates 2000; Boase 2001; Reinhardt and Siva-Jothy 2007; Ter Poorten and Prose 2005; Doggett et al. 2012).

Our analyses integrating microsatellite markers and *kdr*-associated genotype frequencies reveal contrasting patterns of genetic diversity within populations and across regions. At the population level, post-hoc tests confirmed that U.S. bed bug populations harbored significantly more alleles per population than European populations. However, this pattern reversed at the regional scale, where Europe exhibited the highest allelic richness, followed by the U.S. and then Canada. This contrast suggests that different population genetic processes may be operating in long-established ancient Old-World populations versus the invasive New World populations.

In North America, extensive admixture appears to contribute to within-population diversity, whereas European populations exhibit stronger genetic structure with less within-population admixture. STRUCTURE analyses indicate that multiple genetic clusters are present across Europe, but individual populations are typically dominated by one or two clusters rather than showing the extensive multi-cluster admixture characteristic of U.S. populations (Fig. 1a,b, 2). This pattern is consistent with more restricted gene flow among European populations, potentially reflecting geographic separation, historical isolation, or demographic stability maintaining distinct regional lineages. Consequently, while individual European populations harbor fewer alleles, differentiation among populations contributes to elevated regional-scale diversity (i.e., high beta diversity). During

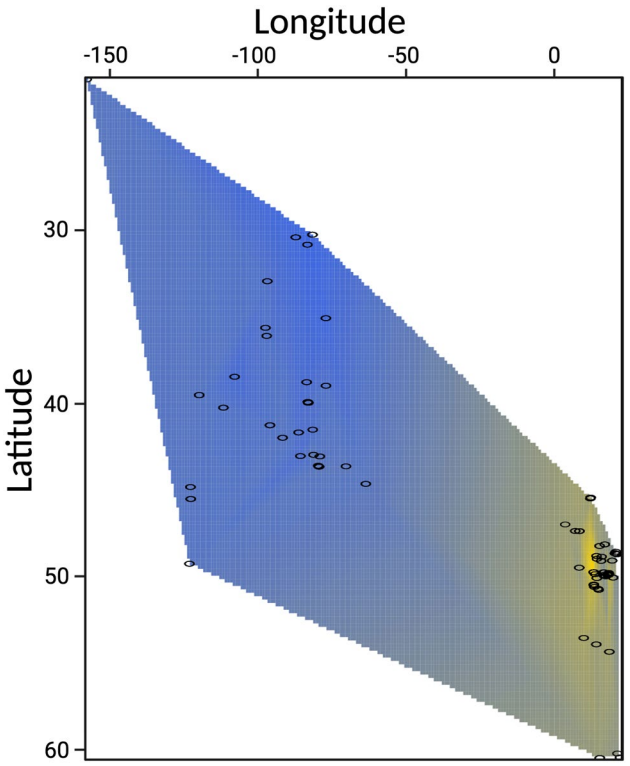

Fig. 4 Spatial Principal Components Analysis (sPCA) illustrating large-scale spatial genetic structure across sampling locations ($n = 109$ populations [USA—35, Europe—65, Canada—9]). Points represent sampling sites projected onto the dominant global spatial component, which captures broad continental differentiation rather than fine-scale isolation by distance. The spatial gradient separating European and North American populations supports a model of region-specific invasion histories shaped by historical bottlenecks and limited post-establishment gene flow

the early resurgence phase, Europe may therefore have functioned as a reservoir of genetic diversity, preserving distinct evolutionary lineages across its geographic range.

Lower within-population diversity in European populations may reflect deeper demographic histories compared to North America. Long-term localized persistence could have allowed genetic drift to reduce within-population diversity while maintaining differentiation among populations. Alternatively, historical insecticide use may have produced localized bottlenecks that reduced diversity within populations while preserving differentiation among survivors (Naylor et al. 2018a; b). In contrast, elevated within-population diversity in U.S. populations is consistent with admixture among historically introduced lineages, potentially predating the modern resurgence. STRUCTURE results indicate substantial ancestry from multiple clusters in most U.S. individuals (Fig. 2), suggesting multiple historical introductions from diverse global sources, potentially during periods of intense international trade in the late 19th and early twentieth centuries. The admixed genetic architecture of contemporary U.S. populations is therefore more consistent with legacy introductions followed by secondary contact within North America than with frequent trans-Atlantic gene flow during the early resurgence period. Consistent with these demographic processes, significant deviations from Hardy–Weinberg expectations were observed across global samples, likely reflecting the combined effects of small effective populations, high levels of within-population inbreeding, recent bottlenecks, and admixture among historical lineages.

The widespread application of synthetic insecticides during the mid-twentieth century dramatically reduced bed bug populations across the U.S. (Romero et al. 2007; Miller 2018) but likely did not achieve complete eradication. Instead, populations may have persisted in cryptic refugia, potentially including settings with limited pest control access, where genetically distinct lineages remained at low densities. Alternative host switching, including to poultry, has been proposed as a reservoir mechanism (Szalanski et al. 2008). However, genetic analyses of poultry-associated populations suggest they largely mirror human-associated populations in both diversity and *kdr* profiles (Saenz et al. 2012; Booth et al. unpublished data). While this does not fully exclude a scenario where rural communities harbored cryptic populations that were subsequently introduced to and reseeded from poultry production facilities, it suggests such mechanisms were unlikely to act as sole reservoirs. More plausibly, cryptic reservoirs may have persisted in settings with limited access to effective pest control, where infestations could remain undetected and untreated for extended periods. The resurgence beginning in the 1990s–2000s, facilitated by insecticide resistance evolution (Romero et al. 2007; Dang et al. 2017), likely enabled expansion of these

Table 2 Distribution and frequencies of knockdown resistance (*kdr*) genotypes in bed bug populations across three geographic regions. Values represent the number of populations with each genotype, with percentages in parentheses

kdr genotype								
Region	No. pops	wild type	L925I	L925Ihet	L925I&V419L	L925Ihet&V419L	L925I&V419Lhet	V419L
USA	33	1 (3.03)	13 (39.39)	0 (0.00)	16 (48.48)	1 (3.03)	2 (6.06)	0 (0.00)
Canada	9	0 (0.00)	9 (100.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Europe	56	2 (3.57)	48 (85.71)	5 (8.93)	1 (1.79)	0 (0.00)	0 (0.00)	0 (0.00)

residual populations and increased opportunities for secondary contact and gene flow.

Canadian populations exhibited intermediate patterns for both microsatellite and *kdr* genotypes. Within-population allelic diversity did not differ significantly from either U.S. or European populations, but regional allelic richness was lowest, likely reflecting the more recent resurgence (Miller 2018). STRUCTURE analyses indicate Canadian populations are dominated by a single cluster (K1) that, while present in both putative source regions, is not the majority cluster in either the U.S. (~ 20%) or Europe (~ 35%). This pattern is more consistent with a strong founder effect and genetic drift acting on a small colonizing population, in line with the reduced allelic richness observed in Canada relative to the other regions, than with direct evidence linking Canadian ancestry to a specific source. In contrast, the absence of the V419L *kdr* mutation in Canada, despite its high frequency in the U.S. (Holleman et al. 2019; Lewis et al. 2023) and its near-absence in Europe (Booth et al. 2015; Balvin and Booth 2018), offers a more specific signal, as this single, strongly selected locus is less susceptible to the stochastic loss expected under drift alone. We therefore interpret the *kdr* profile as suggestive of a European-influence origin of Canadian populations, while the STRUCTURE-based K1 dominance more likely reflects a general founder effect rather than ancestry specifically tied to either source region.

The STRUCTURE patterns observed here align with the invasion and resurgence history of *Cimex lectularius*, characterized by severe bottlenecks followed by rapid, human-mediated expansion (Booth et al 2012, 2018; Saenz et al. 2012; Fountain 2014; Akhoundi et al. 2015; Narain et al. 2015; Talbot et al. 2017; Castex et al. 2025). The dominance of single clusters within regions suggests that contemporary infestations largely derive from a limited number of successful founding lineages. Such patterns are consistent with founder effects and genetic drift in recently established populations with limited subsequent admixture. Importantly, STRUCTURE clusters should not be interpreted as discrete evolutionary lineages but rather as genome-wide signals of shared demographic history consistent with previously inferred invasion pathways (Pickrell and Pritchard 2012; Lawson et al. 2018).

European populations exhibit higher admixture across clusters, including substantial representation of K2, which is rare in North America. This heterogeneity likely reflects more complex demographic histories within Europe and the long-term persistence of divergent lineages, corresponding with the Old-World origin of the species (Usinger 1966). European cities likely function as both sources and sinks, with dense and interconnected urban centers facilitating secondary contact via regional travel and commerce, particularly in the early years following the formation of the European Union and the collapse of the Iron Curtain. The apparent separation between dominant North American clusters further suggests partially independent invasion pathways into the United States and Canada, rather than a single pan–North American expansion. This interpretation is consistent with historical records of regional reintroductions following mid-twentieth century suppression (Miller 2018). Collectively, these results support a model in which modern *C. lectularius* populations reflect the combined effects of severe bottlenecks, rapid demographic expansion, and recurrent long-distance dispersal driven by human movement at regional scales (Booth 2024). Regional differences in cluster dominance and admixture likely reflect variation in introduction history, demographic stability, and connectivity among infestations rather than simple isolation by distance and are consistent with limited evidence for frequent contemporary intercontinental gene flow.

Early-phase sampling likely reduced the opportunity for extensive interregional admixture to erode genetic differentiation. Thus, limited signals of transcontinental gene flow do not imply a complete absence of human-mediated transport but rather suggest that such movement may have been insufficient to overcome strong genetic drift in newly established populations. In this context, Europe's higher diversity and admixture likely reflect legacy populations and repeated introductions predating or coincident with resurgence, whereas North American populations reflect rapid expansion from a limited number of successful founders, potentially from cryptic pre-resurgence-era refugial populations.

Implications for insecticide resistance evolution and spread

The inferred population structure has compelling implications for understanding the global emergence and spread of insecticide resistance in *C. lectularius* (Booth 2024). The dominance of a single genetic cluster within the U.S. suggests that *kdr*-associated resistance alleles likely rose to high frequency within a relatively narrow genetic background before spreading continent-wide via human-mediated dispersal. This interpretation is consistent with previous reports of widespread resistance, reduced genetic diversity, and limited population structure across U.S. infestations (Zhu et al. 2010; Booth et al. 2012; Saenz et al. 2012; Narain et al. 2015; Holleman et al. 2019; Lewis et al. 2023). Resistance evolution in North America therefore appears consistent with strong selection acting on small founding populations followed by rapid demographic expansion, rather than repeated independent origins followed by extensive gene flow. The genetic homogeneity associated with K3 (Fig. 2) supports the hypothesis that resistance alleles spread via demographic expansion of successful resurgence lineages.

At the intercontinental scale, *kdr* mutation profiles are notably dissimilar. The L925I mutation is widespread across

regions (~97% U.S., ~96% EU, 100% Canada), whereas V419L shows strong regional differentiation. Except for two U.S. populations (Zhu et al. 2010), the V419L single mutant has not been detected despite extensive surveys across the U.S. and Europe (Booth et al. 2012; Booth et al. 2015; Dang et al. 2015; Palenchar et al. 2015; Balvín and Booth 2018; Holleman et al. 2019; Lewis et al. 2023; Castex et al. 2025, Yu et al. 2026). In contrast, the compound L925I/V419L double mutant occurs at high frequency in the U.S. (~48%) but remains rare in Europe (~2%). This pattern is consistent with microsatellite data indicating limited intercontinental gene flow and may suggest a North American origin of the V419L mutation.

Conclusion

Across multiple analytical frameworks, our results reveal strong continental-scale genetic structure in *C. lectularius* populations from the United States, Canada, and Europe. Populations present during the early stages of the species' resurgence appear dominated by region-specific genetic lineages shaped by founder effects and rapid demographic expansion following historical introductions. Despite the species' capacity for human-mediated movement, there is limited evidence for frequent contemporary intercontinental gene flow. Instead, dispersal appears highly effective within regions, promoting genetic homogenization across large geographic areas, while intercontinental exchange up to this period has been comparatively rare. Because samples were collected during the first decade of the modern resurgence, the observed genetic patterns primarily capture early invasion and expansion dynamics rather than long-term equilibrium processes and present-day genetic structure. Now, fifteen years after the original samples were collected, and almost 25 years post reemergence onset, the analysis of contemporary populations using newly available whole-genome and chromosome-level genomic resources (Benoit et al. 2016; Miles et al. 2025b) will enable direct comparison with the genetic patterns described here, allowing robust assessment of how population structure has shifted during continued global expansion. Together, these advances provide a genomic framework for tracking spread, monitoring resistance evolution, and supporting evidence-based management of this globally resurgent urban pest.

Author contributions

WB, ELV, and CS designed the experiment. WB, LSM, OB and CDL collected and analyzed the data. WB wrote the manuscript. All authors contributed to the revision of the manuscript and gave final approval for publication.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10340-026-02089-z.

Acknowledgments We thank the numerous pest management companies that provided bed bug samples and Rick Santangelo for the management of the NCSU housed samples.

Funding Funding was provided by competitive grants from the USDA NIFA (AFRI 2008–35302–04565), NSF (DEB-1754394), Oklahoma Center for the Advancement of Science and Technology (HR13-211 and HR20-142), the National Pest Management Foundation, and by grants of the Ministry of Education, Youth and Sports of the Czech Republic no. SVV2013-267 201, InterAction no. LTAUSA18032 and the Grant Agency of Charles University no. 2010/45610. Additional support was from the Joseph R. and Mary W. Wilson Urban Entomology Endowment at Virginia Tech (WB), the Blanton J. Whitmire Endowment at North Carolina State University (CS), and the Urban Entomology Endowment at Texas A&M (ELV). We thank all who contributed to the collecting of material used in the study. Unfortunately, they are too numerous to be mentioned.

Data accessibility Raw data used in this manuscript are available at [https://github.com/lindsaymiles/ClectMsatAnalyses].

Code availability Code is available at https://github.com/lindsaymiles/ClectMsatAnalyses

Declarations

Conflict of interest We declare no competing interests.

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