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Host association drives genetic divergence in the bed bug, *Cimex lectularius*

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Abstract

Genetic differentiation may exist among sympatric populations of a species due to long-term associations with alternative hosts (i.e. host-associated differentiation). While host-associated differentiation has been documented in several phytophagus insects, there are far fewer cases known in animal parasites. The bed bug, *Cimex lectularius*, a wingless insect, represents a potential model organism for elucidating the processes involved in host-associated differentiation in animal parasites with relatively limited mobility. In conjunction with the expansion of modern humans from Africa into Eurasia, it has been speculated that bed bugs extended their host range from bats to humans in their shared cave domiciles throughout Eurasia. *C. lectularius* that associate with humans have a cosmopolitan distribution, whereas those associated with bats occur across Europe, often in human-built structures. We assessed genetic structure and gene flow within and among populations collected in association with each host using mtDNA, microsatellite loci and knock-down resistance gene variants. Both nuclear and mitochondrial data support a lack of significant contemporary gene flow between host-specific populations. Within locations human-associated bed bug populations exhibit limited genetic diversity and elevated levels of inbreeding, likely due to human-mediated movement, infrequent additional introduction events per infestation, and pest control. In contrast, populations within bat roosts exhibit higher genetic diversity and lower levels of relatedness, suggesting populations are stable with temporal fluctuations due to host dispersal and bug mortality. In concert with previously published evidence of morphological and behavioural differentiation, the genetic data presented here suggest *C. lectularius* is currently undergoing lineage divergence through host association.

Keywords: ancestral host species, Cimicidae, host-associated differentiation, host–parasite, speciation

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Introduction

Discerning modes of speciation is a keystone in understanding biodiversity and mechanisms of evolution. Parasitic organisms are particularly informative, with speciation events potentially driven through the development of reproductive barriers between populations associated with alternative host organisms, that is alloxenic speciation (Mehlhorn 2008). This mode of speciation likely stems from local adaptations of the parasite and shifts in its host specificity (Poulin 2007). Local ecological adaptation and differentiation can lead to the formation of host races within a species and is considered

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a major route for sympatric speciation (Coyne & Orr 2004). Host races are defined by groups within a species that (i) use different hosts and exhibit host fidelity; (ii) coexist in sympatry; (iii) feature genetic differentiation at more than one locus; (iv) exhibit a correlation between host choice and mate choice; and (v) feature some level of mutual gene flow (Dres & Mallet 2002). Because members of host races are in general more fit on natal hosts than on alternative hosts, and they produce hybrids with reduced fitness, an eventual discontinuity in gene flow caused by physical isolation or assortative mating may lead to incipient speciation. Often reported in phytophagous organisms [e.g. (Feder *et al.* 1988; Dres & Mallet 2002)], a paucity of examples describing host-associated differentiation and the emergence of host races exists among animal parasites (Marchetti *et al.* 1998; Als *et al.* 2002; McCoy *et al.* 2003; Kempf *et al.* 2009, 2011). Recently however, based upon mitochondrial DNA sequences and morphological and behavioural differences, host-associated lineages were suggested to have developed in a geographically widespread human pest, the bed bug *Cimex lectularius* (Balvín *et al.* 2012a; Wawrocka & Bartonička 2013).

Cimex lectularius is a member of the family Cimicidae, a speciose group of obligate blood-feeding insects (Usinger 1966; Henry 2009). They spend most of their time in the shelter of the host and feed directly on the host (Usinger 1966; Bartonička & Růžičková 2013). Lacking wings, active dispersal appears limited to within buildings, and passive host-mediated dispersal likely shapes dispersal patterns (Balvín *et al.* 2012b; Booth *et al.* 2012; Saenz *et al.* 2012; Fountain *et al.* 2014). Bats are considered the ancestral zoophilic host of *C. lectularius* (Horváth 1913), and the documented association of bed bugs with humans dates back to ancient Egypt (Panagiotakopulu & Buckland 1999). But the association with humans likely is much more ancient, going back to the time when humans and bats sheltered together in caves (Usinger 1966). Common prior to WWII, *C. lectularius* was nearly eradicated in developed countries during the 1940s and 1950s due to the widespread use of DDT and other control measures (Boase 2001). However, in recent years, an unprecedented global resurgence has occurred, likely facilitated by the evolution of insecticide resistance, increased national and international travel, global commerce, and local proliferation of thrift and second-hand shops [e.g. (Pinto *et al.* 2007; Zhu *et al.* 2010)].

A recent study by Balvín *et al.* (2012a) based on mtDNA and morphometric analyses suggested considerable genetic and morphological divergence exists between European *C. lectularius* associated with humans and those collected within the roosts of synanthropic bats. Morphological differences suggest adaptation to alternative hosts, mainly changes associated with sensory, feeding and dispersal needs. Only a single mitochondrial haplotype was shared between human- and bat-associated bed bugs from a total of 20 different haplotypes identified (14 from bats, 7 from humans). Limitations associated with divergence dates based solely upon molecular data notwithstanding (Arbogast *et al.* 2002), Balvín *et al.* (2012a) proposed that the two lineages diverged approximately 245 000 years ago (95% confidence interval 98 696 to 866 522 years ago). Even in situations where humans and bats could reside in the same building, the two bed bug populations appear to maintain host fidelity and thus lineage divergence. This is supported by a recent transplant experiment in which *C. lectularius* collected from humans or bats fed less frequently and had higher mortality on the non-natal host (Wawrocka & Bartonička 2013). While mtDNA has proved valuable in revealing ancestral associations (Avise 2000), it often lacks the resolution to inform us of contemporary gene flow and fine-scale genetic structure. Variation at nuclear DNA loci, on the other hand, can be a more powerful means for detecting population structure and gene flow.

Divergent selection pressures on bat- and human-associated *C. lectularius* should also lead to polymorphisms at selected loci, depending on the extent of contemporary gene flow between the two lineages. Because human-associated bed bugs are extensively treated with insecticides, whereas bat-associated bugs are not, genes that confer resistance to insecticides should differentiate between the two lineages and serve as signatures of lineage divergence. Certain mutations in the voltage-gated sodium channel, which result in *knock-down resistance* (*kdr*) and thus reduced sensitivity to DDT and pyrethroid insecticides (Yoon *et al.* 2008; Zhu *et al.* 2010), are expected to be widespread among human-associated bed bugs – as shown in the United States (Zhu *et al.* 2010) – and absent in *C. lectularius* associated with bats.

In the present study, we assess contemporary gene flow and examine the degree of genetic differentiation of *C. lectularius* populations associated with bats and humans using three classes of genetic markers: microsatellites, mtDNA sequence data and *kdr* haplotypes. Our findings demonstrate that two host-associated lineages exist and experience little contemporary gene flow, despite a lack of ecological barriers. We conclude that there are two genetically divergent host-associated races of *C. lectularius* which may represent an early stage in sympatric speciation.

Materials and methods

Sample collection and DNA extraction

A total of 756 individual *C. lectularius* were collected from human dwellings ($n = 569$ specimens from 66

locations, in 9 countries) and bat roosts (n = 187 specimens from 37 locations, in 9 countries), across 13 European countries (Table S1, Supporting information). All of the bat roosts sampled were within the attics of human-built structures. Specimens were preserved in 96% ethanol. Genomic DNA was extracted from half of the thorax and legs of individual insects using the DNeasy Blood & Tissue kit (Qiagen) and then stored at −18°C prior to use.

MtDNA sequencing and population-genetic analyses

A 658-bp fragment of the cytochrome oxidase subunit I (COI) gene was amplified in 372 specimens representing all 103 locations (Table S1; Fig. S1, Supporting information), using barcoding primers LepF (5′-ATT CAA CCA ATC ATA AAG ATA TNG G-3′) and LepR (5′-TAW ACT TCW GGR TGT CCR AAR AAT CA-3′) (modified from Hajibabaei *et al.* 2006). Additionally, a 382-bp fragment of the 16S rRNA gene was amplified using primers LR-J-13007 and LR-N-13398, according to Szalanski *et al.* (2008). PCR protocols and bidirectional sequencing of PCR products followed those outlined in Balvín *et al.* (2012a). Sequence alignments were performed using MAFFT (Katoh *et al.* 2009). As no incongruence between the two studied genes was detected following a partition homogeneity test (Farris *et al.* 1995) using PAUP* (Swofford 1999), concatenated alignment of both mtDNA fragments (total 1040 bp) was used for further analyses. A median-joining network was constructed, following the algorithm of Bandelt *et al.* (1999) and the rationale of Huson *et al.* (2010), in Network 4.516 (www.fluxus-engineering.com, accessed on 28 May 2013), using default parameters of the program to visualize the data.

Knock-down resistance: partial sequence of the sodium channel gene

Partial or complete *kdr* genotypes were determined across 19 bat-associated and 49 human-associated bed bug collections, that is locations from which ≥3 specimens were available (Table S1; Fig S2, Supporting information). The methodology followed that outlined by Zhu *et al.* (2010). Fragments were amplified in three individuals from each collection site and PCR products mixed according to the intensity of bands on 2% agarose electrophoresis gels (1x TBE). The PCR products were purified using QIAquick® PCR Purification Kit (Qiagen) and sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems) or through a commercial sequencing service (Macrogen Inc.). Chromatograms were aligned using CodonCode Aligner 3.0 (CodonCode Corporation). We scored sequences at two locations known to be associated with *kdr* resistance: amino acid 419 (wild type, GTC = valine; mutation, CTC = leucine) and amino acid 925 (wild type, CTT = leucine; mutation, ATT = isoleucine) (Table S1, Supporting information). Heterozygotes were identified through the presence of overlapping peaks at each specific nucleotide.

Microsatellite DNA genotyping and analyses

Depending on the analysis, samples were divided into two groups: (i) all specimens and (ii) sites containing ≥7 specimens (human dwellings: n = 55 locations, 525 total specimens; bat roosts: n = 14 locations, 130 total specimens) (Table S1, Supporting information). Samples were genotyped at 20 polymorphic microsatellite loci (*BB6B*, *BB15B*, *BB28B*, *BB31B*, *BB38B*, *BB42B*, *Clec6*, *Clec10*, *Clec11*, *Clec15*, *Clec37*, *Clec45*, *Clec48*, *Clec90*, *Clec91*, *Clec96*, *Clec97*, *Clec98*, *Clec99* and *Clec104*), following protocols outlined by Booth *et al.* (2012).

Prior to population-genetic analysis, MICRO-CHECKER version 2.2.3 software (Van Oosterhout *et al.* 2004) was used to assess the presence of null alleles, scoring error, or large-allele dropout across loci. Summary statistics (mean number of alleles, observed heterozygosity) were calculated using Genetic Data Analysis (GDA) software (Lewis & Zaykin 2001). Hardy–Weinberg exact tests were performed using GENEPOP version 4.0 (Raymond & Rousset 1995; Rousset 2008). Bonferroni correction for multiple tests was applied. The latter two tests were performed on samples for which ≥7 specimens were available, with each specimen being genotyped.

To examine potential genetic structuring between hosts and/or locations, the Bayesian clustering algorithm implemented in STRUCTURE version 2.2.3 (Pritchard *et al.* 2000) was performed. Under this method, individuals are probabilistically assigned to a given genetic cluster (K) based on the proportion of their genome that matches that cluster. To determine the true K-value, ΔK (Evanno *et al.* 2005) was implemented. Because of high relatedness of individuals within locations [a consequence of the population establishment process, see Booth *et al.* (2012)], single specimens from each location were used for STRUCTURE analysis to avoid overestimation of the true K-value (Vonholdt *et al.* 2010). STRUCTURE was initially run to determine whether host association influenced genetic structure. STRUCTURE analysis was performed using the admixture model with allele frequencies correlated. Runs were based on 200 000 iterations after an initial 50 000 burn-in period of the Markov chain. K was set to from 1 to 5 to account for both host races and then additional clusters to accommodate further substructure that might exist. Each run was

replicated three times to check for concordance of the data. Δ*K* was determined using the STRUCTURE HARVESTER version 0.56.3 software (Earl *et al.* 2011). Following the identification of *K*, 10 independent runs were then performed at that optimal Δ*K* value. Pr matrices generated during each replication run were aligned using the program CLUMPP version 1.1.1 (Jakobsson & Rosenberg 2007) under the GREEDY algorithm with 10 000 random permutations. Following the detection of division based on host species (see Results), STRUCTURE was rerun within each to detect further population subdivision. Parameters followed those described earlier, with *K* set from 1 to 10. A factorial correspondence analysis (FCA), as implemented in the program GENETIX v4.05.2 (Belkhir *et al.* 1999), was used to further examine the degree of population substructuring among both host species.

Based on the Δ*K* value of 2, genetic boundaries between host clusters were determined using GENELAND (Guillot *et al.* 2005). The Bayesian algorithm implemented in the software is based on a geographically constrained model that takes into consideration the spatial location of individuals screened for a number of microsatellite loci. The inference algorithm for the spatial model was run with the following parameters: (i) number of populations set to 2; (ii) number of iterations = 100 000; (iii) thinning = 100; (iv) uncertainty of coordinate = 0.1; and (v) correlated allele frequency model. Consistency of resulting inference was checked by comparing parameter estimates from 20 independent runs of GENELAND. Single specimens randomly chosen from each population were used in this analysis.

Following the determination of the most likely *K*-value, overall and pairwise F_{ST} [θ analogue: (Weir & Cockerham 1984)] and relatedness (*r*) were calculated in FSTAT v.2.9.3.2 (Goudet 1995). Only locations consisting of ≥7 individuals were used in these analyses. To test for the presence of private alleles among bat- and human-associated bed bugs, GDA software (Lewis & Zaykin 2001) was used employing all samples.

Results

Mitochondrial network analysis

In total, unambiguous sequences from 99 human-associated specimens across 48 localities and 115 bat-associated specimens from 29 bat roosts were analysed (Fig. 1; Table S1, Supporting information). This represents 57.5% of total samples sequenced. The remaining 42.5% of samples (representing 21.6% of all bat-associated samples and 28.8% of all human-associated samples) revealed ambiguous nucleotide positions, consistent with mitochondrial heteroplasmy (G. A. Robison, O. Balvin, E. L. Vargo, C. Schal & W. Booth, Under Review), evident in both COI and 16S genes. No significant sequence homology was found following a BLAST search of the primers or the amplified products against the bed bug genome (NCBI BioProject PRJNA167477) (performed on 23 September 2014); thus, sequence ambiguities were not considered to result from NUMTs. Ambiguous sequences were therefore excluded from further analysis. On the basis of 28 variable sites in COI and nine in 16S, we resolved 24 haplotypes (Table 1). Evaluation of the distribution of haplotypes between human and bat hosts revealed 15 haplotypes present within individuals collected from bat roosts and 11 within human-associated individual bed bugs. The resulting haplotype network (Fig. 2) revealed two major haplogroups: one of bat-associated bed bugs and one of human-associated bed bugs. Only two haplotypes (H13 and H16) appeared 'misplaced' in the alternate haplogroup, and only two haplotypes within the human haplogroup (H2, H25) were shared between collections from bats and humans.

Knock-down resistance haplotype variation

Complete *kdr* genotypes were available for 13 bat-associated collections, with additional six collections failing to amplify an unambiguous PCR product at the 419 region for sequencing, despite several attempts. All 13 bat-associated collections exhibited the wild-type form (GTC = valine) at amino acid 419, and also the wild-type form (CTT = leucine) at amino acid 925 (Table S1, Supporting information). From the human-associated collections, a single location exhibited the mutant form at amino acid 419 (CTC = leucine), whereas the remaining 48 possessed the wild-type form. At amino acid 925, however, 45 (92%) exhibited the mutant form (ATT = isoleucine), two exhibited the wild-type form, and two revealed overlapping peaks at this region for both the mutant and the wild-type form, thus informing us that these had a mix of wild-type and mutant specimens within single populations and/or heterozygous individuals (Table S1, Supporting information).

Population-genetic structure and differentiation across Europe

Across the 20 polymorphic microsatellite loci screened, no evidence was found to support the presence of null alleles, scoring error, or large-allele dropout within the data set. Allelic diversity across all samples ranged from 2 to 29 per locus (mean, 12.25) with observed heterozygosity from 0.048 to 0.276 (mean, 0.166) (Table 2). Splitting samples by host association revealed greater allelic diversity and mean observed heterozygosity within the bat-associated samples [bat: 2–26 (mean

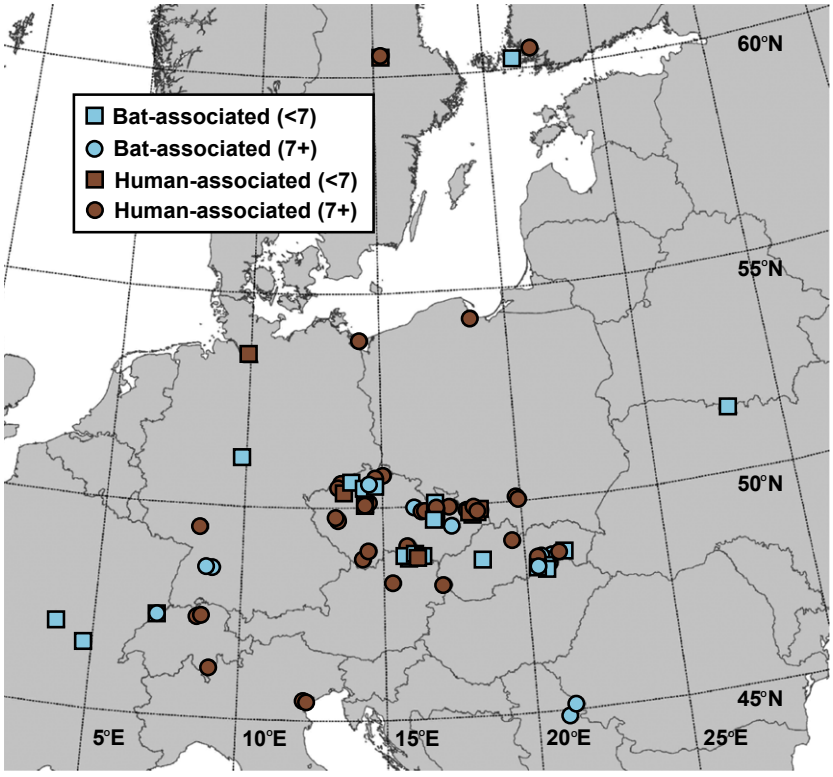

Fig. 1 Sampling locations for *C. lectularius*. Brown (dark) icons represent human-associated samples; blue (light) icons represent bat-associated samples. Circles represent sample sizes of seven or more bed bugs; squares, under seven.

10.5), H_o – 0.306; human: 2–16 (mean 7.5), H_o – 0.130] (Table 2). Private alleles were present in both bat- and human-associated populations, but in general, more were observed in bed bugs collected from bat roosts (average of 4.75 private alleles per locus) than in human-associated samples (1.75 per locus) (Table 2). When samples were separated by host, 9 of 14 (64.3%) bat-associated populations exhibited ≥4 alleles at one or multiple loci, in contrast to only 1 of 55 (1.81%) human-associated samples (Table S1, Supporting information). When all samples were combined, significant deviation from Hardy–Weinberg equilibrium was observed, suggesting population subdivision existed among the sampled locations. Reanalysis by host type, as supported by STRUCTURE results (below), again revealed significant deviations at all loci in both bat- and human-associated *C. lectularius* populations, suggesting the existence of further population subdivision.

STRUCTURE analysis produced a ΔK peak at a $K = 2$ when considering the data set with no *a priori* assumptions of structure. The two clusters cleanly corresponded to the two hosts, bats and humans (Fig. 3). These results were verified following FCA, with samples preferentially clustered with the host from which they were collected (Axis 1 – 18.13%; Axis 2 – 10.11%; Axis 3 – 9.06%). When samples were then grouped following STRUCTURE-assigned clusters, separation was more pronounced (Fig. 4). Results from GENELAND, which can detect population structure in relation to geographic and genetic information, supported the existence of two main genetic clusters associated with bats and humans. Members of these genetic clusters showed no clear geographic associations, and instead exhibited a patchy distribution (Fig. S3, Supporting information), with members of each of the two genetic clusters existing in close geographic proximity to each other.

Overall F_{ST} was 0.683 [95% confidence interval (CI) 0.664–0.701]. Within samples collected from bat roosts alone, overall F_{ST} was 0.468 (95% CI 0.423–0.519), and from samples collected from human dwellings F_{ST} was calculated as 0.718 (95% CI 0.700–0.734). Given the lack of overlap in 95% CIs of F_{ST} values, populations associated with humans were significantly more differentiated than their bat-associated counterparts. Relatedness values within populations followed a comparable trend with overall r estimated as 0.778 (95% CI 0.763–0.792). Within bat roosts alone r was 0.590 (95% CI 0.547–0.633), whereas within human dwellings r equalled 0.805 (95% CI 0.789–0.821), indicating that individuals within human-associated populations were significantly more related than those within bat-associated populations.

Discussion

The present study supports the existence of two host-associated races in *C. lectularius* based on significant genetic divergence of populations on two sympatric

Table 1 Haplotypes and polymorphic sites of mtDNA COI/16S concatenated sequences of *Cimex lectularius* collected from two alternative hosts (human or bat)

Haplotype	Host	COI Accession #	16S Accession #	Nucleotide positions																					
				25	34	37	112	244	265	277	289	293	302	322	331	343	346	352	367	391	433	442	475	481	493
H2	Bat/ Human	GU985525.1	KJ937974	A	T	G	T	C	T	C	G	G	A	G	G	C	C	G	C	G	A	C	G	A	A
H3	Bat	GU985526.1	KJ937969	·	·	A	·	·	C	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
H4	Bat	KJ937979	KJ937969	·	·	A	·	·	C	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
H6	Human	GU985526.1	KJ937974	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
H7	Bat	GU985526.1	KJ937971	·	·	A	·	·	C	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
H8	Bat	KJ937983	KJ937969	·	·	A	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
H10	Bat	KJ937980	KJ937969	·	·	A	·	·	C	·	·	·	·	·	·	·	T	·	·	·	·	·	A	·	·
H11	Human	KJ937988	KJ937976	·	·	A	·	·	·	·	·	·	·	A	·	·	·	·	T	A	·	·	·	·	·
H13	Human	KJ937989	KJ937969	·	·	A	·	·	·	·	A	·	·	·	·	·	·	·	·	·	G	·	·	·	·
H14	Human	GU985523.1	KJ937975	·	·	A	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·
H15	Bat	KJ937986	KJ937969	G	·	A	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
H16	Bat	KJ937987	KJ937968	·	C	A	·	·	·	T	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·
H17	Bat	KJ937980	KJ937970	·	·	A	·	·	C	·	·	·	·	·	·	·	T	·	·	·	·	·	A	·	·
H18	Bat	GU985526.1	KJ937972	·	·	A	·	·	C	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
H19	Bat	KJ937985	KJ937969	·	·	A	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	T	·	·	·
H22	Human	KJ937990	KJ937974	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G
H23	Bat	KJ937981	KJ937969	·	·	A	·	·	C	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
H24	Bat	GU985526.1	KJ937973	·	·	A	·	·	C	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
H25	Bat/ Human	GU985525.1	KJ937977	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
H26	Human	KJ937991	KJ937974	·	·	·	·	T	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	G	·
H28	Human	KJ937984	KJ937978	·	·	A	C	·	·	·	·	A	·	·	A	T	T	A	·	·	·	·	A	·	·
H35	Human	KJ937992	KJ937974	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	G	·
H36	Human	GU985525.1	KJ937968	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
H37	Bat	KJ937982	KJ937969	·	·	A	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·

Haplotype	Host	COI Accession #	16S Accession #	Nucleotide positions															
				511	536	547	595	625	628	680	843	848	849	883	903	932	1007		1008
H2	Bat/Human	GU985525.1	KJ937974	A	C	T	T	G	G	A	G	C	A	C	G	G	G		A
H3	Bat	GU985526.1	KJ937969	·	·	·	C	·	A	G	A	·	·	·	·	·	·		·
H4	Bat	KJ937979	KJ937969	·	·	·	C	A	A	G	A	·	·	·	·	·	·		·
H6	Human	GU985526.1	KJ937974	·	·	·	·	·	·	·	·	·	·	·	·	·	·		·
H7	Bat	GU985526.1	KJ937971	·	·	·	C	·	A	G	A	·	·	·	·	T	·		·
H8	Bat	KJ937983	KJ937969	·	·	·	·	·	·	G	A	·	·	·	·	·	·		·
H10	Bat	KJ937980	KJ937969	·	·	·	C	·	A	G	A	·	·	·	·	·	·		·
H11	Human	KJ937988	KJ937976	·	·	·	·	·	·	·	·	T	·	·	A	·	·		·

Table 1 *Continued*

Haplotype	Host	COI Accession #	16S Accession #	Nucleotide positions												
				511	536	547	595	625	628	680	843	848	849	883	903	932	1007	1008
H13	Human	KJ937989	KJ937969	.	.	C	C	.	.	G	A	.	.	.	.	.	.	.
H14	Human	GU985523.1	KJ937975	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
H15	Bat	KJ937986	KJ937969	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.
H16	Bat	KJ937987	KJ937968	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.
H17	Bat	KJ937980	KJ937970	.	.	.	C	.	A	G	A	.	.	.	A	.	.	.
H18	Bat	GU985526.1	KJ937972	.	.	.	C	.	A	G	A	.	G	.	.	.	.	.
H19	Bat	KJ937985	KJ937969	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.
H22	Human	KJ937990	KJ937974	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H23	Bat	KJ937981	KJ937969	.	T	.	C	.	A	G	A	.	.	.	.	.	.	.
H24	Bat	GU985526.1	KJ937973	.	.	.	C	.	A	G	A	.	G	T	.	.	.	.
H25	Bat/Human	GU985525.1	KJ937977	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
H26	Human	KJ937991	KJ937974	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H28	Human	KJ937984	KJ937978	.	.	.	.	A	.	G	.	.	.	.	.	.	.	G
H35	Human	KJ937992	KJ937974	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H36	Human	GU985525.1	KJ937968	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.
H37	Bat	KJ937982	KJ937969	.	.	.	C	.	A	G	A	.	.	.	.	.	.	.

host species; all three genetic marker classes screened support this conclusion, as does a previous morphometric analysis (Balvín *et al.* 2012a). The mtDNA network appears essentially identical to that of Balvín *et al.* (2012a), showing two partially overlapping clades with bed bugs separated by host race (Fig. 2). In addition to the shared haplotype (H2) reported by Balvín *et al.* (2012a), we identified a second shared haplotype (H25), differing from H2 at a single nucleotide. STRUCTURE (Fig. 3) and FCA (Fig. 4) analyses of microsatellite data support our conclusion that contemporary gene flow between the host races is negligible. Geographic isolation can be excluded as a factor promoting reproductive segregation given the broad geographic overlap of samples collected (Figs 1, S3, Supporting information). When no *a priori* information was considered (i.e. host type or geographic location), samples clustered preferentially by host association, with two exceptions. These exceptions were human-associated bed bugs that aligned with the bat-associated cluster with greater than 60% genetic affiliation (Fig. 3). These samples shared mtDNA haplotype H2, one of two found commonly in both bat- and human-associated *C. lectularius*. Additionally, one sample exhibited a mixed *kdr* haplotype with profiles found primarily in association with bats, and the other in association with humans. These infrequent exceptions to host race differentiation may represent evidence of recent introgression, ancestrally shared alleles, homoplasy, or quite possibly incorrect assignment of dispersing bed bugs to the proper host. Regardless, it appears that *C. lectularius* parasitizing humans are following an evolutionary trajectory essentially independent of those found to parasitize sympatric bats.

The genetic data presented here lend support to the hypothesis that the ancestral host of *C. lectularius* was bats, with one or more human lineages diverging following the movement of humans out of shared cave domiciles (Usinger 1966). Subsequent divergence of the founding human-associated populations can be reinforced through selection for specialization on humans, as suggested for triatomine bugs (Schofield *et al.* 1999). Significantly greater allelic diversity was observed in the bat-associated populations, despite the larger sample size of the human-associated populations. Bed bugs derived from bat roosts were also found to have approximately 2.7 times more private alleles than bugs associated with humans. Combined, these results suggest an ancestral genetic bottleneck in the human-associated lineage, which might be expected following divergence of a small founder population during host transition (Mayr 1963). Indeed, recent independent genetic analyses of human-associated *C. lectularius* populations in the United States and United Kingdom

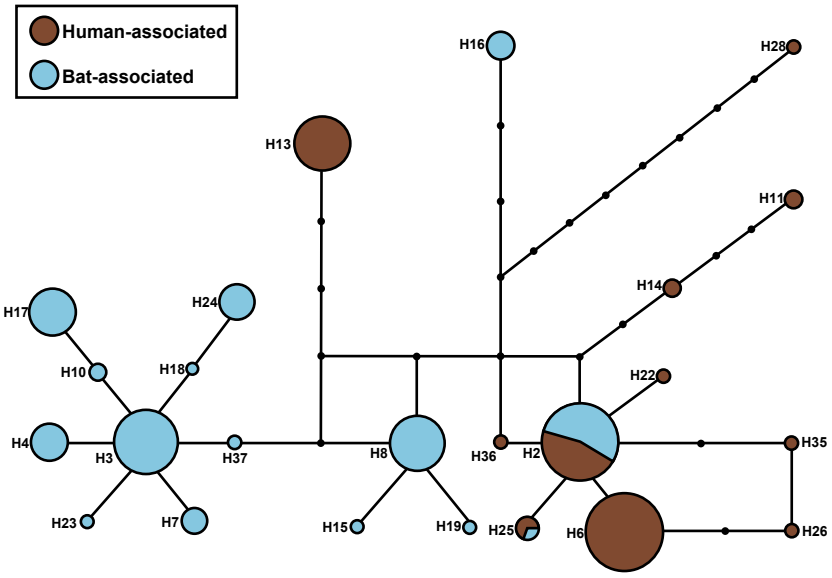

Fig. 2 Haplotype network of human- and bat-associated bed bug samples based on concatenated mitochondrial cytochrome oxidase subunit I and 16S rRNA gene sequences (total length 1040 bp).

support the idea that infestations are founded by small propagules (Saenz *et al.* 2012; Fountain *et al.* 2014). Thus, when viewed in the context of a host shift, the isolation of a small founding propagule during the emigration of humans from caves that they co-inhabited with bats might be expected to produce such a pattern.

In addition to the genetic divergence observed between host-associated lineages, our data provide a contrasting picture regarding infestation dynamics of the two host races in Europe. From European human-associated bed bugs, we see complementary patterns to those reported in the United States – four or fewer alleles per microsatellite locus – suggesting population establishment through the introduction of a single male and single female, singly mated female, or highly related and inbred individuals (Booth *et al.* 2012; Saenz *et al.* 2012; Fountain *et al.* 2014). Only a single sample (1.81%) exhibited more than four alleles (five alleles at a single locus). Bat-associated *C. lectularius*, in contrast, exhibited ≥4 alleles at several loci in 64.3% of sampled locations (9 of 14). The genetically depauperate nature of the human-associated populations may also provide insight into the stability of bed bug populations. Population establishment, high population turnover and extinction events are expected to be common with little opportunity for population admixture, likely due to human-mediated movement and frequent interventions through pest control. Thus, human-associated bed bugs appear to live in highly structured metapopulations. In contrast, *C. lectularius* populations within bat roosts may be expected to be more stable, albeit with temporal fluctuations due to weather events, bug mortality and host dispersal, with multiple introduction events resulting from the latter. Similar to what has previously been reported (Booth *et al.* 2012; Saenz *et al.* 2012), relatedness estimates suggest significant inbreeding events are common in *C. lectularius*; however, the greater allelic diversity present within bat-associated populations results in lower relatedness values within this group.

Results from screening *kdr*-associated mutations provide a somewhat contrasting picture to that previously reported in human-associated *C. lectularius*. Approximately 85% of *C. lectularius* samples collected across the east coast and south-central United States were found to possess either one or both of the *kdr* target-site mutations [haplotypes B, C and D according to (Zhu *et al.* 2010)]. Similarly, 95.9% of bed bugs we collected from human-associated populations in Europe possessed either one or both of the *kdr* mutations, with haplotype B also the most common (wild-type valine at position 419, leucine to isoleucine mutation at position 925). Bed bugs possessing both mutations (haplotype C) appear underrepresented in the European samples (2%) compared to US samples [41%, (Zhu *et al.* 2010)]. Intriguingly, all bat lineages of *C. lectularius* exhibited wild-type amino acids at both positions (haplotype A), which is exceptionally rare among human-associated populations in the United States and in our human-associated European samples. Extensive use of DDT and pyrethroid insecticides within human-built structures was likely selected for *kdr* mutations (Usinger 1966; Snetsinger 1997), thus supporting a lack of contemporary gene flow between these two host races over the last ~60 years. These results are consistent with the hypothesis that the *kdr* haplotype observed in bat-associated samples represents the natural, ancestral haplotype, whereas the human-associated haplotype has been subject to anthropogenic selection with synthetic insecticides.

Table 2 Host-associated locus summary statistics

	Number of alleles					Observed heterozygosity		
		Bat		Human				
Locus	All	Observed	Unique	Observed	Unique	All	Bat	Human
BB28B	23	20	8	15	3	0.203	0.43	0.147
BB38B	9	8	3	6	1	0.170	0.288	0.140
BB31B	24	21	11	13	3	0.229	0.466	0.168
Clec11	6	5	2	4	1	0.235	0.359	0.204
Clec6	2	2	0	2	0	0.048	0.023	0.054
BB42B	20	16	8	12	4	0.227	0.393	0.184
Clec37	8	8	3	5	0	0.156	0.389	0.097
BB15B	29	26	13	16	3	0.191	0.45	0.121
Clec48	3	3	1	2	0	0.098	0.383	0.025
Clec45	3	2	0	3	1	0.095	0.142	0.083
Clec90	12	10	5	7	2	0.201	0.200	0.202
Clec91	11	8	2	9	3	0.102	0.111	0.100
Clec96	14	11	7	7	3	0.142	0.358	0.084
Clec97	8	8	3	5	0	0.197	0.275	0.180
Clec98	14	12	7	7	2	0.220	0.396	0.175
Clec99	8	4	0	8	4	0.203	0.168	0.203
Clec104	13	11	6	7	2	0.092	0.297	0.040
Clec105	20	19	12	8	1	0.276	0.477	0.225
BB6B	16	14	4	12	2	0.111	0.197	0.089
Clec15	2	2	0	2	0	0.121	0.318	0.069
Mean	12.25	10.5	4.75	7.5	1.75	0.166	0.306	0.13

Additionally, our results may shed light on the current global resurgence of *C. lectularius* among humans. The *kdr* profiles of the European populations were different from those in the United States, and thus, it is possible that neither population serves as a significant contemporary source for the other.

While evidence for differentiation is apparent by host race, structure based on geographic region is absent within both host lineages. As bat-associated bed bugs had been collected from the roosts of multiple bat species (see Table S1, Supporting information for a list of species), the sample size did not permit a species-specific analysis; bed bugs from all bat species sampled did however cluster within the bat-associated lineage, but no evidence for further host-associated differentiation among bat species was evident. Furthermore, multispecies roosts were present within the collections. The absence of any discernible population structure within these samples may reflect the movement of bats among roosts, especially in mixed species overwintering roosts (Rehak & Gaisler 1999; Smirnov *et al.* 2007). Although mark–recapture studies with *Myotis myotis* reveal female philopatry (Horáček 1985; Berková *et al.* 2013), parasite infestation can cause host dispersal behaviour (Moore 2013), and switching roosts has been shown to reduce the load of *Cimex* spp. in bats of the genus *Pipistrellus* (Bartonička & Gaisler 2007; Bartonička & Růžičková 2012, 2013). In human-associated bed bug, no geographic structure was evident. This lack of structure within *C. lectularius* derived from human dwellings has previously been reported in samples collected in the eastern USA (Saenz *et al.* 2012) and the UK (Fountain *et al.* 2014) and likely results from extensive human-mediated movement.

The best documented studies of host race formation come from phytophagous insects [e.g. see Mullen & Shaw (2014) for a recent review]. For example, following the introduction of domesticated apples, the apple maggot fly *Rhagoletis pomonella* host-shifted from hawthorn to apple, with changes in oviposition preferences, mating behaviours and host fidelity resulting in disruption of reproduction between host-associated strains, as detected using molecular markers (Feder *et al.* 1988). Surprisingly, however, little evidence is available regarding host-associated genetic differentiation of animal parasites. A recent worldwide population-genetic study of *Aedes aegypti* mosquitoes using polymorphic microsatellites from 24 populations in 13 countries concluded that one genetic cluster included all domestic (anthropophilic) mosquito populations outside Africa and a divergent cluster included both domestic and forest (zoophilic) populations within

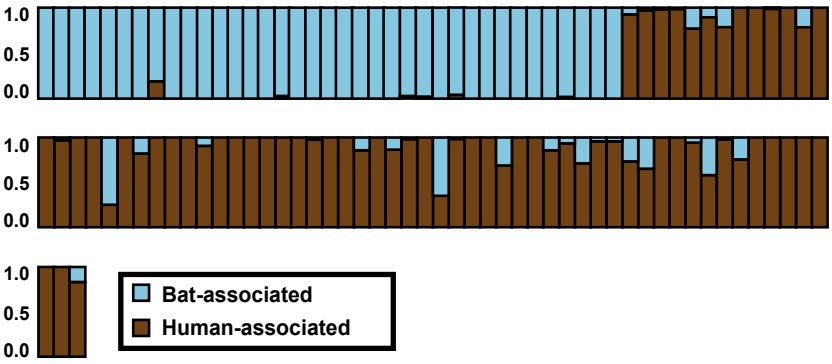

Fig. 3 STRUCTURE plots depicting Δ*K*. Coloured bars represent the proportion membership of each individual bed bug to one of two genetic clusters ($K = 2$). Blue (light) cluster sampled from bat roosts; brown (dark) cluster represents bed bugs associated with humans.

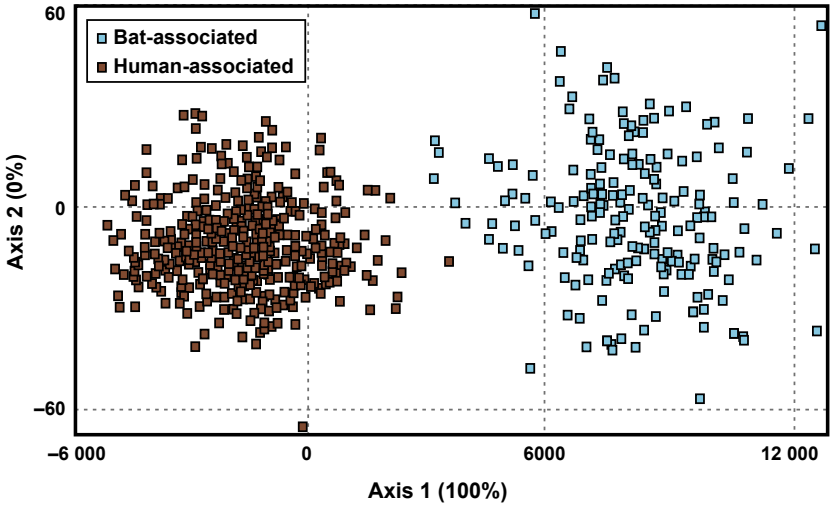

Fig. 4 Results of factorial correspondence analysis showing genetic differentiation based on microsatellite allele frequencies for individual *C. lectularius* collections sampled across Europe. Samples clustered by host: brown (dark) squares represent human-associated samples; blue (light) squares represent bat-associated bed bugs.

Africa (Brown *et al.* 2011). An African origin for ancestral *Ae. aegypti* populations was supported by higher genetic diversity (heterozygosity and private allelic richness) in Africa than outside of Africa. Moreover, the results suggested two domestication (host shift to humans) events: one in Africa and a second domesticated form spread outside of Africa (Brown *et al.* 2011). We document a similar genetic divergence of *C. lectularius* into zoophilic and domestic/anthropophilic lineages that cluster well with morphometric differentiation, host association, host preferences and host fidelity. Whereas African populations of *Ae. aegypti* differentiate along an ecological landscape into forest and urban forms, the two *C. lectularius* races may have the potential to coexist sympatrically in very close proximity within the same building, but with no apparent gene flow between them. Although we have no evidence that both races currently coexist, the persistent resurgence of human-associated bed bugs in Europe escalates the potential for their co-occurrence, as populations increase in size and their European distribution widens.

The presence of strong genetic differentiation between bed bug host races in the absence of geographic separation may identify *C. lectularius* as a unique model system for the study of sympatric speciation and the landscape ecology of potential pathogen transmission. Whereas similarly investigated parasitic species are generally highly mobile and live outdoors, or have relatively large outdoor reservoirs (McCoy *et al.* 2003; Kempf *et al.* 2009, 2011), *C. lectularius* lives strictly 'indoors' in tight dependence on its host for both feeding and dispersal. Given that bats frequently roost within human-built structures, the two *C. lectularius* host races have the potential to occur very near to each other and to their respective hosts. As in other systems of host race formation, in instances where both races coexist, reproductive and ecological isolation may potentially be promoted between the *C. lectularius* lineages because of conceivably strong selection against migrants. Bats and humans have opposite diel (day: night) activity patterns, so a host shift could affect the ability of bed bugs to feed undetected. Migration across host lineages may further be selected against by

reduced preference to feed on the alternative host, and lower viability of bed bugs who feed on alternative host blood, as demonstrated under laboratory conditions by Wawrocka & Bartonička (2013). Ultimately, morphological differentiation associated with sensory, feeding and dispersal behaviours, as observed between these two *C. lectularius* lineages (Balvín *et al.* 2012a), is likely to promote host fidelity and inhibit mixing between the host races.

Finally, this unique system should facilitate investigations into phenotypic and genotypic changes that adapt ectoparasite populations to anthropophilic habits, as well as the potential for pathogen transmission between alternative hosts (e.g. bats, birds) and humans. We hypothesize that the ancestral association with and preadaptation to bats may facilitate a re-association of some human-adapted bed bugs with bats, and even a broadening of their host range to include both hosts. While we have no current evidence of the coexistence of both races within the same building, the current patterns of resurgence across Europe along with instability of the human-built environment (host availability, pest interventions), compared to natural bat roosts, may further facilitate some spatial mixing between these two host races. Because bat-associated bugs could be differentially competent to harbour and transmit pathogens, these commensal interactions should be further investigated.

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W.B. and O.B. designed and performed the research and analysed the data. W.B., O.B., J.V. and E.L.V. supplied the reagents. W.B., O.B., E.L.V. and C.S. wrote the manuscript.

Data accessibility

DNA sequences: GenBank Accession nos for mtDNA haplotypes at COI and 16S are available in Table S1 (Supporting information). Concatenated sequences can be found at Dryad doi: 10.5061/dryad.qf53d. *kdr* genotypes from pooled samples are listed in Table S1 (Supporting information). Microsatellite genotypes: Complete microsatellite genotypes, factorial correspondence analysis genetic input file (GENEPOP format), and GENELAND genotype and geographic coordinate files are available at Dryad doi: 10.5061/dryad.qf53d.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 European sampled locations of *C. lectularius* that were screened for mtDNA.

Fig. S2 European sampled locations of *C. lectularius* that were screened for *kdr*-associated mutations.

Fig. S3 GENELAND generated map of posterior probabilities of population membership (based on microsatellite data) and spatial locations of genetic discontinuities for populations of *C. lectularius* associated with humans (top) and bats (bottom).

Table S1 Sample collection information for *C. lectularius* collected on two alternative hosts, humans and bats.