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# Mitochondrial DNA and morphology show independent evolutionary histories of bedbug *Cimex lectularius* (Heteroptera: Cimicidae) on bats and humans

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**Abstract** The bedbug, *Cimex lectularius*, is a well-known human ectoparasite that is reemerging after a long absence of several decades in developed countries of North America and Western Europe. Bedbugs' original hosts were likely bats, and the bedbugs are still common in their roosts. Using morphometry and sequences of mitochondrial cytochrome oxidase subunit I and 16S genes, we showed that the populations on bats and humans are largely isolated and differ in morphology. The character of the morphological difference suggests it to be due to adaptation to different hosts, namely adaptations to different sensory, feeding, and dispersal needs. Using the molecular data, we estimated the time of splitting into bat- and human-parasitizing groups using the isolation-with-migration model. The estimate is surprisingly long ago and seems to predate the expansion of modern human from Africa. The gene flow between bat- and human-parasitizing bedbugs is limited and asymmetric with prevailing direction from human-parasitizing populations to bat-parasitizing populations. The differentiation of the populations fits the concept of host races and supports the idea of sympatric speciation. Furthermore, our findings contradict recently formulated hypotheses suggesting bat roosts as a source of bedbug's resurgence as a human pest. Also, we extend the known host range of the bedbug by two bat species.

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## Introduction

Studies of host specificity and related phenotypic diversity are crucial for understanding the evolution of species diversity and variability of life strategies in parasitic organisms. True generalist parasites must be able to occupy different environments and to cope with their various hosts' specific defenses. Specialization for particular hosts may result in formation of host races characterized by fidelity to different hosts and sympatric occurrence at the same time, host-associated genetic differentiation, and restricted but appreciable mutual gene flow (Dres and Mallet 2002). Such a process of differentiation of a parasitic species may culminate in speciation, i.e., evolution of an array of closely related parasitic species each specialized for a particular host. The phenomenon of host races is regarded as one of the fundamental arguments supporting the concept of sympatric speciation (Coyne and Orr 2004). The formation of host races has been repeatedly demonstrated in phytophagous organisms (for references, see Dres and Mallet 2002), but very few examples are found among animal parasites (Als et al. 2002; Langmore et al. 2008; Marchetti et al. 1998; McCoy et al. 2003). Parasite populations and particularly those of animal parasites experiencing the initial stages of isolation are thus very important from the viewpoint of evolutionary biology. The study of their genetic and phenotypic

differentiation is crucial in disentangling the causations of new species' emergence.

The bedbug, *Cimex lectularius* Linnaeus, 1758, is likely the most widely known member of the order Heteroptera. It is an example of a parasite known to exploit several sympatric hosts with highly different ecology (mostly bats, humans, some other mammals, and birds) (Eichler 1937; Usinger 1966). Since some of the host shifts were probably relatively recent (Povolný and Usinger 1966; Usinger 1966), the bedbug represents an optimal model for a study on initial stages of host specialization. Furthermore, study of the bedbug as a reemerging human pest (e.g., Doggett et al. 2004; Hwang et al. 2005; Reinhardt and Siva-Jothy 2007; Romero et al. 2007; Reinhardt et al. 2008; Zhu et al. 2010) is particularly topical, and understanding its biology is essential for its control.

*C. lectularius* is a member of the family Cimicidae containing nearly 100 species parasitizing mostly bats and birds. Besides *C. lectularius*, two other species from the family are associated both to bats and man: *Cimex hemipterus* (Fabricius, 1803) and *Leptocimex boueti* (Brumpt, 1910). The diversity of cimicid taxa associated with birds and their phylogenetic position within bat-associated cimicids points to multiple independent evolution of association with birds and suggests that the bats are the original host of the family as well as of the ancestor of the three human-associated species. Since the bats are frequent hosts of all the three species even recently and both bats and humans often shared caves as shelters, this is usually accepted as the most likely hypothesis (e.g., Usinger 1966; Horváth 1913).

As an alternative hypothesis, Weidner (1958) suggested that human-associated *C. lectularius* evolved from populations hosted by pigeons (*Columba livia*), namely from the *Cimex columbarius* Jenyns, 1839, the sister species to *C. lectularius* (Ueshima 1964; Usinger 1966). Nevertheless, *C. columbarius* is reproductively isolated from *C. lectularius* (Ueshima 1964), while the human-associated and bat-associated populations of bedbugs were shown to be able to

fully interbreed (Usinger 1966). Thus, even if we accept that *C. lectularius* evolved from *C. columbarius*, the independent evolution of the human-associated and bat-associated populations of bedbugs from *C. columbarius* is extremely unlikely.

The bedbug is definitely the most generalist parasite among cimicids. Most records from the free-living hosts other than bats were summarized by Eichler (1937) (tit, starling, redstart, blackbird) and Dubinij (1947) (marmot, pika, vole, wagtail, lark). Nevertheless, most of the recorded host species are found among domestic or synanthropic animals, such as poultry, rats, pigeons, swallows, martins, or sparrows. Moreover, considering the anecdotic nature of most of these records, none of the recorded hosts except bats and pigeons are likely to play an important role in the origin of the bedbug as a human pest.

The bedbug was recorded in bat roosts in Europe and few other regions: Afghanistan (Povolný and Usinger 1966), Iraq (Abul-Hab 1979; Lanza 1999), and Kyrgyzstan (Rybin et al. 1989). The record from Afghanistan is the only finding of the bedbug in a cave, i.e., in a natural, non-synanthropic environment. Povolný and Usinger (1966) concluded that this finding could represent an autochthonous bat-parasitizing population of the bedbug. In Europe, the bedbug often occurs in bat colonies roosting in buildings. However, it seems absent in southern ranges of their host bat species where they roost in caves that are usually too cold for cimicids (Simov et al. 2006). The species of bats recorded as bedbug hosts are listed in Table 1.

If the bats were the original host of the bedbug, the likely original distribution of the bedbug would be Palearctic (Usinger 1966) and humans acquired the bedbug when they shared caves with bats as shelters (e.g., Usinger 1966; Horváth 1913). Due to its association with human beings, the distribution of *C. lectularius* is today nearly cosmopolitan (Usinger 1966).

The only published population-genetic study on the bedbug is based on the 16S mitochondrial ribosomal subunit gene and covered 22 populations from man and poultry from the USA, Canada, and Australia (Szalanski et al.

**Table 1** Bat species recorded as hosts of the bedbug

| Bat species | Previous and our records |
| --- | --- |
| *Pipistrellus kuhlii* (Kuhl, 1817) | Abul-Hab 1979 |
| *Pipistrellus pygmaeus* (Leach, 1825) or *P. pipistrellus* (Schreber, 1774) | New host record |
| *Vespertilio murinus* Linnaeus, 1758 | Dubinij 1947 |
| *Myotis mystacinus* (Kuhl, 1817) | Poppius 1912 |
| *Myotis myotis* (Borkhausen, 1797) | e.g., Povolný 1957; Usinger 1966; confirmed |
| *Myotis blythii* (Tomes, 1857) | Usinger 1966; confirmed |
| *Myotis daubentonii* (Kuhl, 1817) | Bogdanowitz 1994; Wagner 1967 |
| *Nyctalus leisleri* (Kuhl, 1817) | Bobkova 2001; Walter 2004 |
| *Myotis emarginatus* (Geoffroy, 1806) | Protić and Paunović 2006; confirmed |
| *Eptesicus serotinus* Schreber, 1774 | New host record |

2008). It revealed large genetic variability even within localities, but particular haplotypes were shared between the individuals from man and poultry. Those authors concluded that there is no evidence that a bottleneck occurred (as some expect) after the bedbugs were practically eradicated in the 1940s and 1950s, and they ascribed this observation to bedbug survival in refugia in poultry or bats at that time and a subsequent switch back to humans.

The present paper is focused on characterizing the relationship between the bedbug populations from bats and humans, the most common hosts nowadays. We discuss the application of host-race concept sensu Dres and Mallet (2002). We comment on the possible schemes of host-association history of the bedbug. Based on the sample available, we test the time since separation of the human- and bat-parasitizing groups. Because the nowadays synanthropy of bats in the studied area often causes both hosts sharing shelters, we estimate the degree of their isolation and the shape of possible mutual gene flow. Since the bedbug was shown to exhibit morphological variation suggested to be caused by actual host association (Eichler 1937; Johnson 1939; Slack 1937; Usinger 1966), we complete the molecular study by thorough morphological analysis. We examine morphological differences between the groups and comment on their possible adaptive significance. In addition, our samples significantly contribute to the knowledge of the host range among bat species; therefore, we review the published data in the light of our results.

Our data contribute to understanding the recent bedbug resurgence as a human pest. We counter the hypothesis pointing to bats as a possible source of bedbug expansions on humans. Also, people's tolerance to bats is a crucial issue in bat protection and the knowledge on the epidemiological threats that bats represent can be highly beneficial.

## Material and methods

### Material studied

The material of 189 bedbug individuals from bats and people used in the study was collected at 91 localities in 14 countries (supplementary table). Individuals from bats were collected either by the authors when accompanying bat specialists on their fieldwork at roosts or by the bat specialists themselves. Some of the material from humans comes from the collections of pest exterminators in the Czech Republic, some—including localities when the host was unclear—were acquired in random collections by the authors' colleagues and acquaintances. All attempts to collect bedbugs from other hosts, namely pigeons, were unsuccessful.

The material was preserved in 96% ethanol and deposited into the collection of Ondřej Balvín at Charles University in Prague. Species determination followed Usinger (1966).

### Morphological analysis

The specimens were photographed in a standardized manner in a Petri dish with ethanol and flattened by a smaller dish using a stereoscopic microscope (Olympus SZX9) and a digital camera (Olympus C-5060) operated by Photo Micro 2.0. The measurements were taken using MeasureIT (Olympus). If not stated otherwise, the largest possible dimension of a body part was measured. In total, 61 characters listed below were measured (abbreviation in brackets, further used in the expression of relative characters). Most of these are illustrated in Fig. 1: head—head width (hw), lengths (al1–4) and widths (aw1–4) of antennal segments, eye width (ew), eye diameter (ed), intra-ocular space dorsally (is) and ventrally (iv), length of hairs between the eye and antenna (se), clypeus width (cw), length of hairs on the clypeus (sc), and lengths (rl1–3) and widths (rw1–3) of rostral segments; pronotum—pronotum width (pw), length (pl), length measured medially (pm), depth of the frontal pronotal concavity (pc), length of hairs (sp); scutellum—scutellum width (sw), length of typical hairs (ss), number of hairs (sn); hemelytra—hemelytra length (hl), width (wh), length of hairs (sh), ratio of length of hairs to their mutual distance on the inner half of the disk of hemelytra (ih); abdomen—length of hairs on the posterior lateral angle of the 2nd–8th tergite (sa2–8), length of hairs on the 9th tergite (sa9), and average length of hairs of the anterior (st3, 5, and 7) and posterior (ts3, 5, and 7) row in the medial third of the 3rd, 5th, and 7th tergum; legs—lengths (fl1–3) and widths (fw1–3) of femora and lengths (tl1–3) and widths (tw1–3) of tibiae.

We analyzed 93 individuals from 47 localities from humans, 78 individuals from 33 localities from bats, and 12 individuals from 5 localities from unknown host. The data were analyzed using Statistica 8.0 software (StatSoft 2007). To explore mutual relationship among all measured characters, we performed a principal component analysis (PCA) using all measured characters. We further analyzed only axes explaining significant portions of variation according to the broken stick model (Jackson 1993). We tested the differences between the two groups of specimens from different hosts by analysis of variance (ANOVA) of factor scores for particular principal components. Then we formed a third group from specimens from unknown hosts and compared it with the two with known hosts by unequal $n$ honestly significant difference (HSD) post hoc tests of the factor scores.

Differences in body shape were also tested for individual characters using analysis of covariance (ANCOVA). Pronotum width, a precisely measurable character highly correlated with overall body size in PCA, was chosen as a covariate in most ANCOVAs. We tested the differences in relative proportions of dimensions within particular body parts or among different articles of legs and antennae or among different leg pairs using the longer, frontal, or proximate
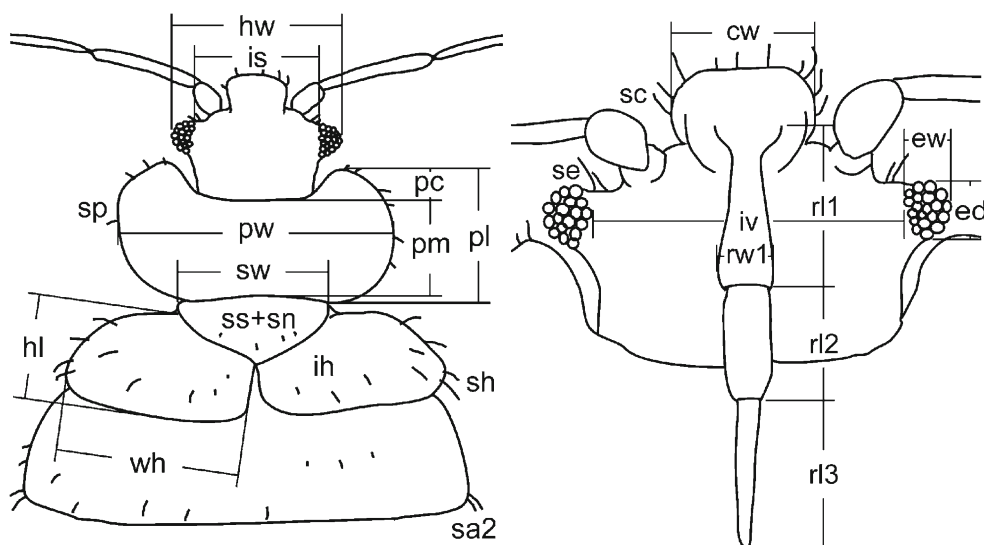
**Fig. 1** Illustration of most characters measured in the morphological analysis. Head width (*hw*), eye width (*ew*), eye diameter (*ed*), intraocular space dorsally (*is*), intraocular space ventrally (*iv*), length of hairs between eye and antenna (*se*), clypeus width (*cw*), length of hairs on the clypeus (*sc*), lengths (*rl1–3*) and widths (*rw1–3*) of rostral segments. Pronotum width (*pw*), total length (*pl*) and length medially (*pm*), depth of the frontal pronotal concavity (*pc*), length of hairs on pronotum (*sp*), scutellum width (*sw*), average length of hairs on scutellum (*ss*) and their approximate number (*sn*). Hemelytra length (*hl*) and width (*wh*), length of hairs on hemelytra (*sh*), ratio of length of hairs on the inner half of the disk of hemelytra and their interval (*ih*), length of hairs on the posterior lateral angle of the 2nd–8th tergite (*sa2–8*)

dimension as the covariate. In cases where interaction of the categorical variable and covariate was not significant, we removed it from the model. Differences in hair lengths were tested by two-group nonparametric Mann–Whitney *U* test.

Moreover, we performed a discriminant function analysis (DFA) using the specimens with known host association. Because we aimed to classify specimens regardless of their body size, in DFA we used characters independent of body size or body dimensions statistically controlled for body size. For this purpose, we created a set of characters using lengths of hairs (they do not increase with body size) and residuals of simple linear regression from the same pairs of characters used in ANCOVAs mentioned above (altogether 119 characters). Then, we performed PCA using this set of characters and chose 29 of them according to their mutual correlations and the ANCOVA results. This elimination procedure allowed us to keep maximum information on differences in body shape and at the same time to minimize the number of variables. These 29 variables were inputs to DFA, where we made further selection of variables useful for specimen classification based on backwards stepwise selection. Using the DFA classification function, we classified the specimens collected from uncertain host to either the bat or human group.

DNA extraction, PCR, and sequencing

We analyzed 59 individuals from 30 localities from humans, 75 individuals from 33 localities from bats, and 12 individuals from 6 localities from unknown host. The tissue for

DNA extraction was obtained from a half of the thorax and legs. Extraction was performed using DNeasy® Blood & Tissue Kit (QIAGEN).

Amplification of the cytochrome oxidase subunit I (hereinafter COI) gene fragment was performed using modified DNA 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′) designed for Lepidoptera (e.g., Hajibabaei et al. 2006). The fragment of the 16S rRNA gene (16S) was amplified following Szalanski et al. (2008) with general 16S primers published by Kambhampati and Smith (1995): LR-J-13007 (5′-TTA CGC TGT TAT CCC TAA-3′) and Simon et al. (1994): LR-N-13398 (5′-CGC CTG TTT ATC AAA AAC AT-3′). The portion of COI sequenced (excluding primers) was 658 bp and the portion of 16S was 382 bp.

The annealing temperature in the PCR was 42°C for both fragments. The PCR products were purified using QIAquick® PCR Purification Kit (QIAGEN). The sequencing was done in both directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems) or using a commercial sequencing service (Macrogen Inc., South Korea).

Alignments and population genetics analyses

Basic population genetic polymorphism analyses (nucleotide and haplotype diversities) were performed with the program Arlequin 3.01 (Excoffier et al. 2005). The sequences were aligned using MAFFT (Katoh et al. 2009). The

congruence of data for the two genes was tested by partition homogeneity test (Farris et al. 1995) using PAUP* 4.0b10 (Swofford 1999). Since the test showed no incongruence between the studied genes ($p=0.55$), concatenated alignment of both mitochondrial fragments was used for further analyses. We constructed a median-joining network (for the algorithm and rationale for using this type of network, see Bandelt et al. (1999) and Huson et al. (2010)) in Network 4.516 (www.fluxus-engineering.com, accessed 10 Sep 2010) using default parameters of the program to visualize the data. Since one haplotype (h12, sample 89) exhibited a large number of unique mutations, it was excluded from all other analyses. We assume the sequence could represent a nuclear paralog of a part of the mitochondrial genome. Since the network suggested existence of two partially separated groups according to host specialization, we examined components of genetic variability at hierarchical levels using analysis of molecular variance (AMOVA) in Arlequin 3.1 (Excoffier et al. 2005). The components of diversity in the hierarchical model were comprised of within localities, among localities/within groups, and among groups. The allocation of localities into groups was based on bedbug presence on particular hosts (humans and bats). In accordance with the result of the discriminant analysis based on morphological characters, 12 specimens from six localities with uncertain host were assumed for purposes of the population genetic computations to be associated with bats. However, we ran the analyses with specimens with known host association only as well.

Inference based on simple interpretation of gene trees is difficult at the population level because the gene trees have a strong random component due to stochastic population genetic processes (Nielsen and Beaumont 2009). New approaches based on coalescent theory which have been recently developed and incorporated in freely available software (known as coalescent genealogy samplers) should be the preferred choice instead of the traditional estimates based on a single tree (Kuhner 2008; Nielsen and Beaumont 2009). We applied the isolation-with-migration model (IM) using the program IMa (Hey and Nielsen 2004, 2007; Nielsen and Wakeley 2001) to estimate parameters for the evolutionary history of the bat and human groups. The program estimates six demographic parameters: the population-split time, the effective population size for the ancestral population and for the current populations, and the migration rates in both directions. The posterior probability densities of the model's parameters are generated by simulating genealogies by Markov chain Monte Carlo method. Random sample of individuals is a necessary precondition of correct program runs and reliable estimates. Since individuals collected at one locality logically cannot represent random sample of population due to possible kin structure and/or inbreeding, we used sequences of single randomly chosen individuals from each locality as input data. We created two alignments: one excluding and one including the specimens with unknown host association. The program was run three times using each alignment in the M-mode with identical settings in order to assess convergence. Each run began with a burn-in period 1,000,000 steps long and continued for 10,000,000 steps. Metropolis coupling was implemented using 20 chains and geometric heat mode. The adequacy of chain mixing was assessed by trend line plots and effective sample size (ESS) values. For all runs, all ESS values were greater than 40,000.

Lacking any other published estimates, we used the "standard" arthropod substitution rate for mitochondrial DNA (1.15%/Ma) as reported by Brower (1994) to scale the parameters of the IM model into the actual population parameters. For scaling the effective population sizes, we had to estimate the number of generations per year. The generation time varies according to temperature from 34 days at 28°C to 236 days (reflecting rather arrested development) at 13°C (Johnson 1941). The other important factors are the availability of host, character of dwelling, and climate. Such number of variables prompted us to set a variable scaling: we scaled the upper confidence limit of effective population sizes by three generations per year, the lower by one and the maximum likelihood estimate by two. We used the log-likelihood ratio test comparing nested models with the best likelihood estimates in order to assess whether the estimates of migration rates significantly differ from zero or the effective population sizes differ from one another.

## Results

### Morphological analysis

Only two principal components, PC1 and PC2, explaining 35.39% and 21.57% of the total variability, respectively, were significant according to the broken stick model. The dimensions of pronotum, scutellum, hemelytra, and head were strongly and negatively correlated with PC1 (Fig. 2). Therefore, PC1 can be interpreted as the expression of the overall size of specimens. However, the head width is correlated also with PC2. Similarly, the dimensions of body extremities (antennae, rostrum, and legs) or hair lengths showed correlations with PC2. Thus, PC2 largely represents differences in hair lengths or body extremities relative to overall body size.

The groups of specimens from different hosts (bats versus humans) significantly differed in factor scores at both axes (ANOVA: PC1: $F=42.24$, $p<0.0001$; PC2: $F=299.1$, $p<0.0001$). The bugs from bats are larger, more hairy, and have relatively shorter extremities.
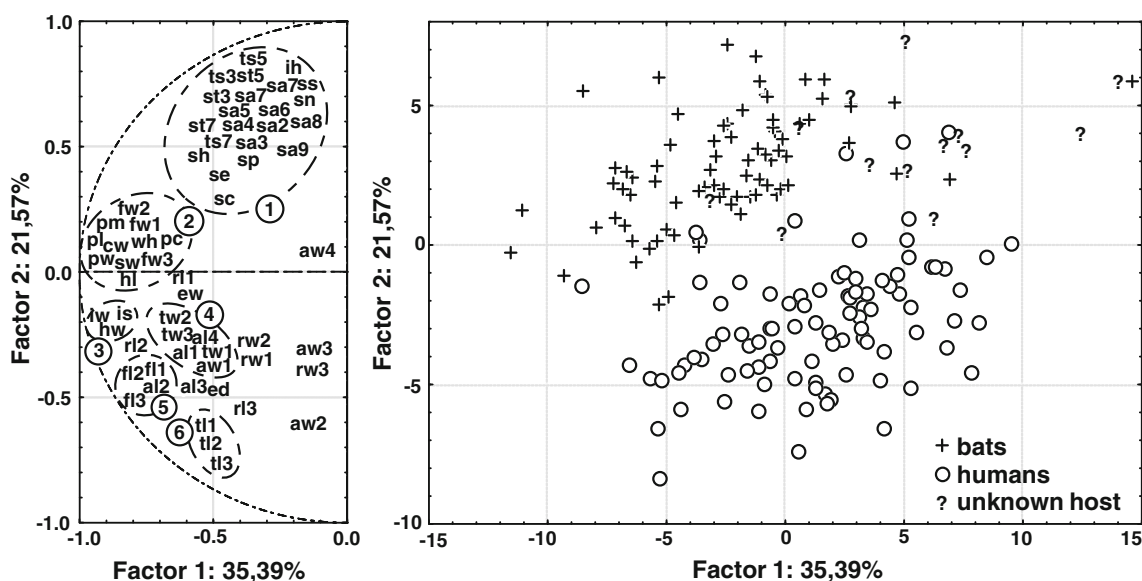
**Fig. 2** PCA based on measured characters: projection of characters (*left*) and specimens (*right*) on the first two PCA axes. Clusters of characters: *1* hairs; *2* dimensions of pronotum, scutellum, hemelytra, and width of femora; *3* dimensions of head; *4* width of tibiae; *5* length of femora; *6* length of tibiae. For abbreviations, see "Material and methods"

The group formed from the specimens from unknown hosts differed from both groups with known hosts along PC1 (unequal n HSD test: humans: $p<0.0001$, bats: $p=0.019$), but only from the group from humans in the division along PC2 (PC2: humans: $p<0.0001$, bats: $p=0.29$; PC3: humans: $p=0.02$, bats: $p=0.09$).

When the differences between groups were tested by ANCOVA, the host association significantly affected the variability of characters in 58 out of 90 comparisons. Interactions between the host association and continuous predictor were removed from the model in all cases. For example, in the specimens from bats, all femora and at least two tibiae were wider in relation to both their length (first to third femur: $F=4.05$, $p=0.046$; $F=108.7$, $p<0.01$; $F=118.4$, $p<0.01$; second and third tibiae: $F=29.27$, $p<0.001$; $F=30.36$, $p<0.001$) and the width of pronotum (first to third femur: $F=6.2$, $p=0.014$; $F=10.3$, $p=0.002$; $F=6.4$, $p=0.013$; first and third tibiae: $F=14.38$, $p<0.001$; $F=17.8$, $p<0.001$) and shorter in relation to the width of pronotum (first to third femur: $F=73.5$, $p<0.001$; $F=72$, $p<0.001$; $F=105$, $p<0.01$; second and third tibiae: $F=238.4$, $p<0.01$; $F=335.2$, $p<0.01$). In general, posterior legs are longer than anterior ones in the bedbug, but the differences in lengths of all tibiae and the posterior two femora are larger in the specimens from humans (second tibia related to first: $F=14.6$, $p<0.001$; third tibia related to second: $F=22.8$, $p<0.001$; third femur related to second: $F=6.9$, $p<0.01$). Also, the tibiae are longer in relation to the length of femora in the specimens from humans, with the differences increasing posteriorly (first to third leg: $F=45.9$, $p<0.001$; $F=130.7$, $p<0.01$; $F=180.1$, $p<0.01$).

Similarly, the ANCOVA calculations showed significant differences in lengths or widths of many antennal or rostral segments. Also, all hairs measured were longer in the specimens from bats (with the exception of ts7, the $p$ values in all tests were under 0.001).

The following characters were included into the discriminant analysis by selection based on backward stepwise model building: sa7, st5, sn, and regression residuals of rl3/pw, rw3/pw, fl2/pw, fw2/pw, tl3/pw, aw2/aw1, rl1/hw, and fw2/fl2 (character used as the continuous predictor after the slash). Based on these characters, the groups of specimens were well discriminated (Wilks' lambda$=0.116034$; $p<0.01$). All specimens with known host association were classified correctly; the posterior probabilities for just two of them were lower than 0.95. Using the discriminant function based on the specimens with known host association, all specimens from unknown host were classified within the group from bats with the posterior probabilities always exceeding 0.95. For the canonical scores of cases, see Fig. 3.

Molecular analysis

Polymorphism statistics for the two mitochondrial genes in bedbugs are summarized in Table 2. The molecular analysis based on the 16S and COI genes revealed 21 mitochondrial haplotypes in 69 localities. Of 51 localities sampled by more than one individual, more than one haplotype was present at only three. Only one haplotype (h2) was shared by both groups from bats and humans. The estimated haplotype network (Fig. 4) suggests the existence of two partially overlapping clades characterized by presence on human
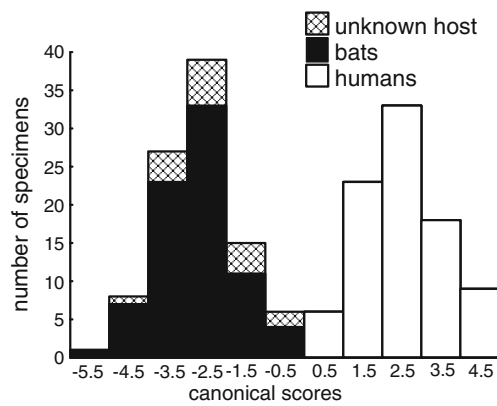
**Fig. 3** Canonical scores from the discriminant function analysis of morphological differences among bedbugs from different hosts

versus bat hosts. The existence of distinct bat and human clades was further confirmed by AMOVA (Table 3). A significant amount of genetic diversity could be attributed to differences among groups based on the association with particular hosts.

Parameters of the IM model describing the partially isolated groups are shown in Fig. 5 and Table 4. Only the results of the first run using the alignment including the specimens with unknown host association are presented, as the other five produced very similar values. The time estimate for the split of the bat- and human-parasitizing groups is rather problematic due to limited information concerning the mutation rate. However, even the estimate based on the lower limit of 90% highest posterior probability density of the time parameter is surprisingly large. The results show very limited gene flow between the two groups and suggest that the gene flow is asymmetric with the prevailing direction from the human-parasitizing group into the bat-parasitizing one. While the likelihood of the nested model with zero migration rate from bats to humans was not significantly lower than the likelihood of the fully estimated model (Table 5), for the opposite migration rate the likelihood of the full model was significantly better. On the other

hand, the likelihood of the model with migration rates equal in both directions was not significantly lower than the likelihood of the estimated model. This probably was due to relatively low estimated values. Using the nested models, we found no significant evidence of either smaller effective population size of the ancestral population (bedbug population before the split of the bat- and human-parasitizing groups) or difference in effective population size of the two groups specialized for different hosts. It should be noted, however, that the likelihood profile characterizing the estimated values of ancestral population size is rather flat (Fig. 5), and thus, the broadly ranging size values obtained have very similar likelihoods and that results in a large confidence interval.

## Discussion

### Recent occurrence of the bedbug on bats

At least in central Europe, *C. lectularius* is very common in the roosts of *Myotis myotis*. Beside the bedbug, *M. myotis* and other bats in the region often host the exclusively bat-associated *Cimex pipistrelli* species complex.

We personally visited total 56 roosts of *M. myotis* in the Czech and Slovak republics and Hungary, sometimes mixed with bats of other species. The number of bats at the roosts varied from almost abandoned to 3,000. Only in two of these we did record no cimicids. Out of the positive records by all collectors, 32 *M. myotis* roosts were infested by *C. lectularius* and 39 by the *C. pipistrelli* group. We never recorded *C. lectularius* and the *C. pipistrelli* group together in a single roost, but they definitely do not vicariate geographically as suggested by Povolný (1959) and K. Hůrka (in litt.). Negative controls by other collectors were not recorded. However, the roosts of *M. myotis* in Germany or Switzerland seem not to be infested by cimicids as often as

**Table 2** Polymorphism characteristics of the bat and human groups; sample 89 excluded

| Gene | Length of sequence | Group | No. of localities | No. of sequences | No. of haplotypes | Polymorphic sites | Haplotype diversity | Nucleotide diversity |
|------|------|------|------|------|------|------|------|------|
| COI | 658 | Bat | 39 | 87 | 11 | 15 | 0.7827±0.0310 | 0.004764±0.002762 |
| | | Human | 30 | 59 | 6 | 11 | 0.6981±0.0517 | 0.003085±0.001953 |
| | | Both | 69 | 146 | 16 | 23 | 0.8196±0.0218 | 0.005452±0.003082 |
| 16S | 382 | Bat | 39 | 87 | 7 | 5 | 0.7200±0.0348 | 0.003195±0.002275 |
| | | Human | 30 | 59 | 4 | 4 | 0.4396±0.0732 | 0.002132±0.001725 |
| | | Both | 69 | 146 | 9 | 6 | 0.6975±0.0249 | 0.003606±0.002474 |
| Both | 1,040 | Bat | 39 | 87 | 14 | 20 | 0.8568±0.0195 | 0.004188±0.002315 |
| | | Human | 30 | 59 | 7 | 15 | 0.7604±0.0432 | 0.002735±0.001622 |
| | | Both | 69 | 146 | 20 | 29 | 0.8643±0.0196 | 0.004774±0.002586 |
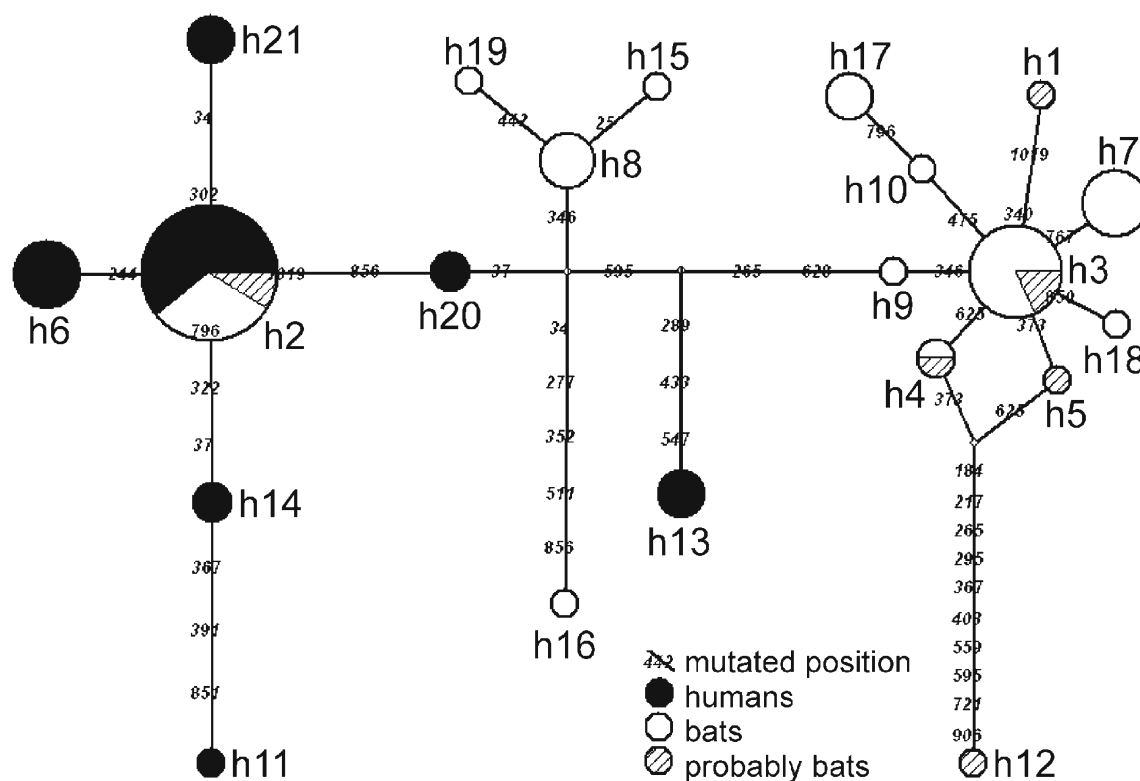
**Fig. 4** Estimated haplotype network of human- and bat-associated bedbug populations based on cytochrome oxidase subunit I and 16S rRNA gene. The populations marked as "probably from bats" with uncertain host association were shown by DFA to be originally from bats

are those in the Czech and Slovak republics or Hungary (Ingo Sheffler, Christian Dietz, Phillipe Christe in litt.).

We confirmed the occurrence of *C. lectularius* in a roosts common to *Myotis blythii* and *M. myotis* (Usinger 1966: south Slovakia; our records: northeastern Hungary, samples 411 and 412). In two roosts inhabited by *M. blythii* exclusively in the same region, we recorded no cimicids. Though this species is biologically and physically very similar to *M. myotis*, it is not known to host any other cimicids and, for some unknown reason, may not represent a suitable host for them.

As far as we are aware from the published data (Table 1), the record of *C. lectularius* from *Eptesicus serotinus* is new (samples 77 and 413, see supplementary table) as well as from *Pipistrellus pipistrellus/Pipistrellus pygmaeus*. Samples 173 and 3 were collected from a country cottage and a hunting stand nearby where bedbugs were biting people, but these buildings were also inhabited by colonies of either *P. pipistrellus* or *P. pygmaeus*. The bugs from the hunting stand (sample 3) carry a unique haplotype (h1) while those from the gamekeeper's house (sample 173) carry the only haplotype (h2) found in both populations from humans and bats. Morphologically, however, both samples appear to be originally from bats.

The most common bat hosts of the bedbug, *M. myotis* and *Myotis emarginatus*, are originally cave-roosting bats, and they began to inhabit buildings only several centuries ago

(Ivan Horáček in litt.). *C. lectularius* is not found in their cave roosts in Europe (Simov et al. 2006), likely because of the climatic conditions in caves. Its only record from a cave was reported from Afghanistan (Povolný and Usinger 1966, bat species not reported); hence, its presence in cave roosts of *M. myotis* and *M. emarginatus* in such warmer areas can be expected. Due to the climatic conditions in the colder parts of the two species' cave dwelling area, there is likely a discontinuity between the possible autochtonous populations in warm caves and the population that inhabits buildings. In this light, the degree of observed haplotype diversity

**Table 3** Hierarchical analysis of molecular variance

| Variance component | Variance | % total | p | Φ-statistics |
|---|---|---|---|---|
| Samples with unknown host association included | | | | |
| Among groups | 1.208 | 38.55 | <0.00001 | $\Phi_{CT}$=0.385 |
| Among localities | 1.734 | 55.34 | <0.00001 | $\Phi_{SC}$=0.901 |
| Within localities | 0.194 | 6.11 | <0.00001 | $\Phi_{ST}$=0.939 |
| Samples with unknown host association excluded | | | | |
| Among groups | 1.341 | 41.66 | <0.00001 | $\Phi_{CT}$=0.417 |
| Among localities | 1.780 | 55.28 | <0.00001 | $\Phi_{SC}$=0.948 |
| Within localities | 0.099 | 3.06 | <0.00001 | $\Phi_{ST}$=0.969 |
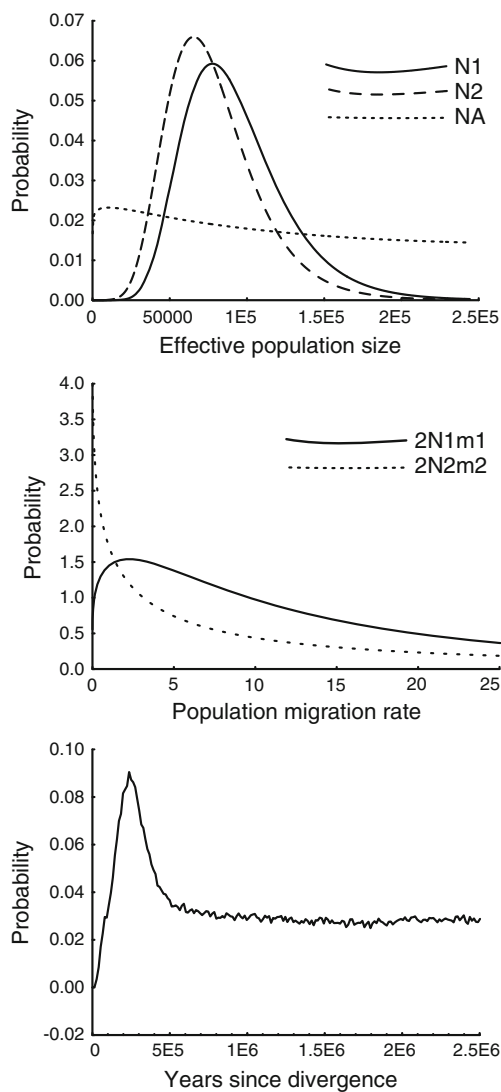
**Fig. 5** Posterior probability distributions of parameter estimates from IM model. The time estimate is scaled using the "standard" arthropod substitution rate for mitochondrial DNA of 1.15%/Ma (Brower 1994)

in the central European population of *C. lectularius* on *M. myotis* and *M. emarginatus* is rather surprising. If we assume this diversity is caused by contacts of *M. myotis* and

*M. emarginatus* with other bat species that roost in tree holes or rock crevices (such as *E. serotinus* or *Pipistrellus* spp.) with higher summer temperatures, such contacts and the transmission of the bedbug between the species must have been frequent.

Do human- and bat-associated populations of *C. lectularius* represent host races?

Both morphological and molecular data show an interesting host-associated differentiation of the population of the bedbug. We believe that the present situation is likely to fit the concept of host races according to Dres and Mallet (2002) that is defined by several conditions. Four of them emphasized as the most important are met in the bedbug: (1) The human- and bat-associated populations use different hosts and the depth of their genetic divergence, estimate of migration rate, and degree of morphological differentiation points to their host fidelity. (2) They are sympatric, and not only in the geographic meaning: They both often inhabit human buildings in the studied area. (3) Though we studied only a single locus, they seem to be genetically differentiated. (4) They undergo a mutual gene flow.

The quantification of mutual gene flow and genetic differentiation is rather problematic due to use of only mtDNA in our study. According to our results of IM model, the migration rate in both directions is far below the limit for distinction of host races and species given by Dres and Mallet (2002). Anyway, the populations cannot be regarded as separate species because they are capable of interbreeding (Usinger 1966).

The condition of spatial replicability of the genetic variation is met at least by the fact that the presence of people in the studied area and probably also bats in part of the studied area (Horáček 1983–4) is more recent than the time estimate of the population split. The character of the morphological differentiation of the populations suggests that the differences are due to adaptations to particular hosts, and thus, we can expect that the condition supposing less fitness on non-natal host is also met.

**Table 4** Maximum-likelihood estimates and 90% highest posterior density intervals of parameters from IM model

|  | $N_1$ | $N_2$ | $N_A$ | $m_1$ | $m_2$ | $2N_1m_1$ | $2N_2m_2$ | $t$ |
|---|---|---|---|---|---|---|---|---|
| MLE | 80,027 | 67,565 | 13,317 | 1.29663E−05 | 2.875E−08 | 2.075 | 0.004 | 245,652 |
| Lower 90% HPD | 42,152 | 32,866 | 122 | 2.5875E−07 | 2.875E−08 | 0.022 | 0.002 | 98,696 |
| Higher 90% HPD | 146,982 | 129,877 | 202,451 | 4.27513E−05 | 3.39538E−05 | 12.567 | 8.820 | 866,522 |

The upper confidence limit of effective population sizes is scaled by three generations per year, the lower by one and the maximum likelihood estimate by two

$N$ effective sizes of the population from humans ($N_1$), from bats ($N_2$), and of the ancestral population ($N_A$); $m_1$ migration rate per year from population from humans into population from bats; $m_2$ migration rate per year from population from bats into population from humans; $2N_1m_1$ population migration rate from population from humans into population from bats; $2N_2m_2$ population migration rate from population from bats into population from humans; $t$ time since population split in years

**Table 5** Log-likelihood ratio tests of nested models

| Model Dataset | log($P$)[a] | 2LLR[b] | log($P$)[a] | 2LLR[b] |
| --- | --- | --- | --- | --- |
| | Including specimens with unknown host | | Excluding specimens with unknown host | |
| 1. $m_1$, $m_2 = 0$ | −6.1893 | −1.8175 | −7.0807 | 0.7180 |
| 2. $m_1 = 0$. $m_2$ | −9.6499 | 5.1036[c] | −10.0755 | 6.7075[c] |
| 3. $m_1 = 0$. $m_2 = 0$ | −10.3488 | 6.5016[c] | −10.3507 | 7.2579[c] |
| 4. $m_1 = m_2$ | −6.9481 | −0.299 | −6.7311 | 0.0187 |
| 5. $N_1 = N_2 = N_A$ | −7.5045 | 0.8130 | −7.4833 | 1.5230 |
| 6. $N_1 = N_A$. $N_2$ | −7.5031 | 0.8102 | 7.4103 | 1.3772 |
| 7. $N_2 = N_A$. $N_1$ | −7.4882 | 0.7803 | −7.4695 | 1.4954 |
| 8. $N_1 = N_2$. $N_A$ | −7.0981 | 0.0001 | −7.1142 | 0.7848 |

Nested models compared to the estimated full model: (1) model in which migration from the population from bats to the population from humans is equal to 0 ($m_1$, $m_2 = 0$); (2) model in which migration the other way is equal to 0 ($m_1 = 0$, $m_2$); (3) model without migration in either direction ($m_1 = 0$, $m_2 = 0$); (4) model in which migration rates are equal in both directions ($m_1 = m_2$). The effective population sizes were the same as for the full model; (5) model in which all population effective sizes are equal; (6) model in which the effective size of the population from bats and the ancestral population are equal; (7) model in which the effective size of the population from humans and the ancestral population are equal; (8) model in which the effective size of the population from bats and from humans are equal. The migration rates were the same as for the full model

[a] Estimates of the posterior density function under the full model

[b] Log-likelihood ratio statistics calculated as the difference between the highest posterior probability for the full model and the highest posterior probability for the nested model

[c] Likelihood of the estimated full model significantly higher than likelihood of the nested model

Our data let us give only limited comments to the remaining characteristics of host races—correlation between host choice and mate choice and lower fitness of hybrids. During our sampling, we have documented several situations of bats bringing bedbugs to people's homes. Nevertheless and despite such situations apparently occurring quite often these days due to the synanthropy of bats, we found only a little genetic evidence of the contact between the populations from bats and humans. This could also be due to specific host or mate selection, less fitness when breeding on nonnative host or less fitness of hybrids. Testing the hybridization of the populations and their survival on non-native host in vivo is an interesting issue for future studies. However, a considerable number of studies on similar ectoparasites occupying different hosts suggest that they readily change even non-related hosts when given an opportunity (e.g., Berrilli et al. 2002; Dick et al. 2009; Krasnov et al. 2007). As a conclusion, we believe the situation in the bedbug that we describe largely falls under the definition of host races by Dres and Mallet (2002), represents a rare example of such situation among non-phytophagous organisms, and thereby is a valuable support for the concept of sympatric speciation.

## The history of human association of C. lectularius

Our data are not able to fully resolve whether the human-associated population originated from the bat-associated one or whether it happened in the opposite way. The distribution of the genus *Cimex*, excluding the human-associated populations, is exclusively Eurasian and Nearctic. The cimicids parasitizing bats in Africa are from different subfamilies (Usinger 1966). The geographic distribution of particular bat species hosting the bedbug (if ever, comprising northwest Africa at most (Aulagnier et al. 2008); paleontological data insufficient) also rather suggests that the switch took place in Europe or Asia, not in Africa where modern humans originated.

The hypothesis suggested by Weidner (1958) finds the origin of the human-associated populations of the bedbug in its sister species *C. columbarius* parasitizing pigeons (see "Introduction"). In this scheme, the bat-associated population of *C. lectularius* originated from the human-associated one. The lower limit of 90% highest posterior density interval of our time estimate of the split of bat- and human-associated populations suggests that humans infected Eurasian bats very soon after they spread from Africa. But if any higher part of the interval is closer to reality, the origin of the bedbug on bats becomes very unclear and it points back to bats as the original hosts. Weidner's hypothesis suggests much more complicated origin of the bat-associated population of the bedbug and comprises larger number of host switches compared to the hypothesis suggesting bats to be the original hosts of the bedbug. Thus, we consider it less likely.

The hypothesis suggesting bats to be the original hosts is usually accepted as the most likely (e.g., Horváth 1913; Sailer 1952; Usinger 1966). Our time estimate of the population split suggests that the bedbug associated with humans as early as the anatomically modern human dispersed out of Africa during the last interglacial period (Armitage et al. 2011). In fact, the maximum likelihood estimate even substantially predates the oldest evidence of anatomically modern human out of Africa (Armitage et al. 2011; Derricourt 2006; Shea 2008). This raises a possibility that modern humans acquired the bedbug from contacts with the earlier inhabitants of the area, namely *Homo neanderthalensis* or *Homo erectus*. Similar scenarios involving direct contact between prehistoric and modern forms of humans have been suggested for other human ectoparasites as well (Reed et al. 2004). The confidence interval of our time estimate is quite wide, however, and the calibration depends on the assumption of a constant mtDNA substitution rate. For example, the substitution rate in different groups of lice was shown to be highly elevated (Light and Reed 2008; Johnson et al. 2003; Hafner et al. 1994) in comparison to the commonly supposed substitution rate in arthropods (Brower 1994) that we

used for our calibration. If bedbugs, which are also human ectoparasites, also have elevated substitution rate, the time estimation of the population split would be closer to the spread of modern humans through Eurasia.

### What was the refugium for the recent resurgence of the bedbug as a human pest?

The systematic development of bedbug control began with the First World War, when the bedbug problem intensified due to large movements of people and further rise of cities. The bedbug population in developed countries was practically eradicated after the Second World War (Usinger 1966). In recent decades, however, it has made a dramatic comeback to once again become an important epidemiological threat. The reasons for its recent resurgence are believed to be the large degree of resistance to several insecticides (Romero et al. 2007; Zhu et al. 2010; Feroz 1969; Kilpinen et al. 2011) and/or the increase in air transport (Reinhardt and Siva-Jothy 2007). Reinhardt et al. (2008) showed, too, that younger generations had lost the ability to identify the bedbug, which delays the onset of control and also may help the bedbug to spread.

Szalanski et al. (2008) suggested bats to be one of possible reservoirs supplying the current global recovery of the bedbug as a human pest. Our findings suggest only occasional switches between the human- and bat-parasitizing groups since their split and that the bedbugs mostly switched from humans to bats. Obviously, unlike bats which can prevent heavy infestation only by changing roosts (Bartonička 2008; Bartonička and Gaisler 2007), people always have possessed some means of exterminating bedbugs, and thus, they often could get rid of them soon after they appeared. Beside the possible specific host choice or less fitness of hybrids discussed above, this could be also a reason for the degree and shape of the mutual gene flow. Anyway, the limited evidence of the contact of the two populations strongly suggests that bats have not served as reservoirs and have not contributed to the current dramatic spread of the bedbug among humans.

Since the study of Szalanski et al. (2008) is based on sequences of 16S only while the present study defines haplotypes using two genes, the comparison is rather problematic. If we define haplotypes using just the 16S sequences, however, the number of haplotypes revealed in the group from humans is smaller in our study than in that of Szalanski et al. (2008) (4 versus 21). Also, the nucleotide and haplotype diversities estimated from the 16S sequences of bedbugs from humans tend to be lower in our study (nucleotide diversity: 0.002132, SD=0.001725 vs. 0.005140, SD=0.003242; haplotype diversity: 0.4396, SD=0.0732 vs. 0.8306, SD=0.0199). Furthermore, three of four haplotypes defined by 16S in our study correspond to the haplotypes

revealed by Szalanski. Hence, it seems that the European bedbug diversity might represent only a derivative of the American diversity. In addition, there are probably only few reports of problems with the bedbug in poultry facilities in Europe (Zbyněk Semerád, Animal Health and Welfare Department, State Veterinary Administration of the Czech Republic in litt.). Thus, as also suggested by Szalanski et al. (2008), we tend to conclude that the global recovery of the bedbug as a human pest has as its source poultry facilities in the USA.

One of the principal challenges to bat protection is the tolerance of people to the presence of bats in buildings. To date, people are seldom aware of the presence of cimicids in bat roosts. Together with the ongoing expansion of the bedbug among humans, there is a risk of this information becoming more widely known and of its decreasing people's tolerance for bats. Therefore, it is important to demonstrate and make publicly known that bats are not to blame for this spread.

### Morphological adaptation of the bedbug to different hosts

We have demonstrated that there are large morphological differences between the groups of bedbug specimens feeding on humans and bats. We believe it is the actual host association that influences the phenotype of the bedbug, as suggested by several authors in the past. Eichler (1937) and Slack (1937) commented on that but gave no evidence. Johnson (1939) showed a difference in the ratio of head width to length of the third antennal segment between *C. lectularius* from animal and poultry housing and from humans. Usinger (1966) described slight changes in this character in bedbugs from man after rearing on bat, chicken, mouse, pigeon, or rabbit. The latter suggests that such differences can be attributed to phenotypic plasticity. The proximate mechanisms responsible for these differences could as well be a local genetic adaptation or genetic drift in the isolated populations, however, and thus they deserve further investigation. We can only speculate on potential adaptive values of these differences.

The differences in leg dimensions could reflect different dispersal needs. Differences in relative leg lengths can correlate with maximum running speed (Full and Tu 1990; Ting et al. 1994). Longer legs in the human-associated population might have developed because the human dwellings are usually concentrated and such legs can be beneficial for active dispersal among houses or apartments (as described, e.g., by Lýsek 1966). They can also help in escaping when detected by humans, while chemical defense is sufficient to avoid being killed by bats (Usinger 1966). Bat roosts are usually scattered through the country, and the bedbug has to rely on clinging to the body of a bat in order to get to another roost. Stronger and shorter legs could be favorable for this purpose, while traveling hidden in people's clothes or baggage does not require strength in legs. The differences in widths and lengths of rostral segments could reflect

different strengths of epidermis for bats and humans, loss of hairs could reflect the different body surfaces of hosts, and differences in dimensions of antennal segments or eyes may answer to different sensory needs.

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