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Phylogeography and ecology of the *Cimex* species (Heteroptera: Cimicidae) in Europe; the evolution of taxa and host specialization

Ph.D. Thesis

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I hereby declare that the present thesis has not been submitted in order to obtain any academic degree earlier or at another institution. My involvement in the research presented in the thesis is expressed through the authorship order of the included publications and manuscripts. All publications and other sources used in the thesis have been properly cited.

In Prague, September 2013

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Abstract

For the complex relationships with their hosts the parasitic organisms are often found to display unusual and interesting modes of diversification and speciation. The species of the genus *Cimex* (Heteroptera: Cimicidae) belong among the obligate ectoparasitic haematophagous groups of insects. Unlike in most other ectoparasites, the strategy of cimicids consists of remaining hidden in the shelter of their host. They use the host body only to feed and disperse. The advantage of the lower competition with other ectoparasites is counterbalanced by the need for particularly stable blood source, for which the cimicids choose social hosts living in colonies. The most frequent and the original hosts of cimicids are bats.

The host range of particular species of Cimicidae is often rather broad. The morphological analysis of the *Cimex pipistrelli* species group showed, however, differentiation according to host bat species. This suggests a need for adaptation to particular host species within the usual range. The differentiation was not found reflected in the mitochondrial DNA. The host associated morphological variability likely caused as many as three species of the *C. pipistrelli* group to be described from Europe, from which two were shown invalid.

Cimicids readily feed on substitute hosts which can be distant from those within their usual host range. However, a long term survival requires a large degree of adaptation which development may not be possible when a continuous gene flow from populations on the original hosts is occurring. Beside bats, the bed bug *Cimex lectularius* parasitizes humans as well. Mitochondrial and nuclear DNA together with morphology showed that the bed bugs from bats and humans follow separate evolutionary trajectories for at least tens of thousands years. Furthermore, despite the synanthropy of the bat species from which samples came, no contemporary gene flow was detected.

The host switches from bats to birds repeatedly led to an increase in biodiversity of Cimicidae. The mitochondrial DNA suggested that the species of the former genus *Oeciacus* associated with birds of the family Hirundinidae each evolved independently by a switch from bats. However, based on both mitochondrial and nuclear data these species were shown to represent an inner group of the genus *Cimex*. The morphological difference between the species of the genus *Cimex* and the former genus *Oeciacus*, visible at the first sight, is thus only a consequence of their different host specialization.

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Published papers and manuscripts:

- Mitochondrial DNA and morphology show independent evolutionary histories of bedbug *Cimex lectularius* (Heteroptera: Cimicidae) on bats and humans (Balvín, O., Munclinger, P., Kratochvíl, L., Vilímová, J. 2012)
- Transport of bugs of the genus *Cimex* (Heteroptera: Cimicidae) by bats in western Palaearctic (Balvín, O., Ševčík, M., Jahelková, H., Bartonička, T., Orlova, M., Vilímová, J. 2012)
- Batbugs (*Cimex pipistrelli* group, Heteroptera: Cimicidae) are morphologically, but not genetically differentiated among bat hosts (Balvín, O., Kratochvíl, L., Vilímová, J. 2013)
- Molecular evidence places the swallow bug genus *Oeciacus* within the bat and bed bug genus *Cimex* (Heteroptera: Cimicidae) (Balvín, O., Roth, S., Vilímová, J. 2013)
- Distribution and host relations of species of the genus *Cimex* on bats in Europe (Balvín, O., Bartonička, T., Simov, N., Paunović, M., Vilímová, J. 2013)
- Host type differentiation drives deep divergence in the common bed bug, *Cimex lectularius* (Booth, W., Balvín, O., Shall, C., Vargo, E.L., Vilímová, J.)
- Indication for frequent paternal inheritance of mitochondria in the bed bug (Heteroptera: Cimicidae: *Cimex lectularius*) (Balvín, O., Vilímová, J.)

Determinants of life strategies and diversity in ectoparasitic insects

Parasitism is a life strategy of many diverse groups of organisms; many of which have reached large evolutionary success. The host-parasite systems are often very complex and diverse in adaptations on both sides of the relationship. For both these reasons such systems are frequent research objects.

The evolutionary success of a particular group can be measured by its species diversity. This is only one side of view but other measures are difficult to be quantifiable. The potentials for diversification or the speciation mode in each group are shaped by many diverse factors. Likely the principal one is the degree of host specificity. On one hand, low host specificity decreases the probability of extinction on one of the hosts and eases speciation via host shift. On the other, high specificity allows for more specialization and more viability and thus less probability of extinction on the specific host (Poulin 2007).

Speciation or diversification potential within an entity of parasite stems from fragmentation of the population. The population structure is shaped by the host range, the population structure of the hosts and the mode of dispersal. The term *infrapopulation* is introduced in parasites covering a population within a single host individual (Esch et al. 1975). The *deme* of a parasite, a randomly mating population more or less isolated from other such populations (Mayr 1963), may be or may not be the same entity depending on the particular host-parasite system (Huyse et al. 2005). Defining the structure of these categories helps predicting the differentiation of the parasite or the mode of speciation.

Parasites with fragmented populations can develop local adaptations to different environment or hosts. Local adaptation can lead to a development of *host races* (Dres & Mallet 2002). The host races are a concept defined by 1) use of different host and host fidelity, 2) coexistence in sympatry, 3) genetic differentiation in more than one locus, 4) correlation between host choice and mate choice and 5) actual mutual gene flow. These characteristics have to be spatially and temporally replicable. The members of host races usually have higher fitness on natal than alternative host and produce less fit hybrids.

The gene flow among host races is not only a condition of a theoretic concept of sub-specific entity. It has been shown that particular rate of migration promotes the local adaptation (Gandon & Michalakis 2002). The probability of fixation or sweep of new characters is tightly connected with the effective size of population (N_e). N_e is tied with the degree of stochasticity in dynamics of the population (Barrett et al. 2008). Size and fluctuation of the parasite population result from the life cycle, fecundity, degree of physical association with the host, mode of dispersal and other features of the parasite and from the availability and capability of the host.

Unification of mating preferences within a host race or similar entity can lead to discontinuity in the gene flow and a rise of a new species, i.e. ecological speciation. The other possibility is founder event speciation via spatial isolation. Selection and physical separation may take different parts in the emergence of a parasite species. The former will be more important if N_e is smaller and the latter when larger (Huysse et al. 2005).

Ectoparasitic lifestyle has developed in many diverse groups of arthropods. Each group has its own characteristics regarding their host exploitation strategy, i.e. modification of life cycle or morphological and physiological adaptations. However, their needs have a lot in common and the adaptive pressures often led to development of convergent characters in different groups. On the other hand, some characters represent constrains for diversification e.g. among broader host range. It has been demonstrated that particular combinations of characters lead to more diversification of an ectoparasitic group than others (Poulin et al. 2009).

The research summarized in the present thesis was aimed to capture the characteristics mentioned above and describe the diversity of a model group of ectoparasitic Heteroptera, the genus *Cimex* Linnaeus, 1758. The following text gives a survey of similar groups, i.e. ectoparasitic insects with life history more or less shaped by physical association with their hosts. Their characteristics are compared with the model group in order to describe the effect of life history of the model group on the diversification and speciation potential in detail and context.

If not cited otherwise, the information is compiled from Marshall (1982), Lehane (2005), Poulin (2007) and Mullen & Durden (2009). For each ectoparasitic group, basic information on diversity, host choice and degree of specificity, life cycle and dispersal modes is provided. Also, studies dealing with their biogeography, phylogeny and population genetics are briefly reviewed or at least sampled.

Diptera: Hippoboscoidea

Hippoboscoidea is a group of obligate blood feeding ectoparasites of endothermic vertebrates. It includes about 200 species and 19 genera in family Hippoboscidae, 150 species and 25 genera in Streblidae and 275 species and 12 genera in Nycteribiidae. They are closely related to Glossinidae with which they share the blood feeding habit and the adenotrophic viviparity producing 3rd instar larvae which are ready to molt to pupal stage.

Streblidae are found to constitute two distinct clades – one from the Old and one from the New World (Dittmar et al. 2006). They are likely paraphyletic with respect to Hippoboscidae and maybe even to Glossinidae. Nycteribiidae are then found to have likely evolved within the Old World clade of Streblidae. Due to the unclear phylogeny and relation to Glossinidae it is not sure what was the ancestral habitat and life style of Hippoboscoidea.

Unlike Glossinidae, adults of Hippoboscoidea are more or less permanently associated with their hosts. They possess short and rather flat body adapted to move on the surface of the host animal. Their legs are remarkably stout and strong. Nycteribiidae are completely wingless; the other two families comprise species with fully developed and with partially or fully reduced wings. Some species of Hippoboscidae lose wings after they locate the host. Halteres are always kept.

Hippoboscidae parasitize variety of different mammals and birds; about 75% species are associated with birds. Among mammals, five orders are known to host Hippoboscidae, however, most host species are found among bovids or cervids. The bird hosts belong to 18 different orders. In contrast, Streblidae and Nycteribiidae are strictly bat related. Considering the probable paraphyly of Streblidae, the association to bats either evolved twice within Hippoboscoidea or Hippoboscidae evolved within bat related flies as a group with broader host choice.

On higher taxonomic levels, the host range of genera of Hippoboscidae comprises either mammals or birds but usually rather smaller taxonomic units. Only a single genus is known to comprise both mammal and bird related species. On species level, Hippoboscoidea vary from monoxenous to generalists but mostly they are highly host specific, especially the species related to mammals (e.g. Maa 1971, Marshall 1976).

The winged species of Hippoboscidae or Streblidae move freely among different host individuals. Others are mostly confined to a single host individual. Nycteribiidae die within several hours off host.

The single larva produced is kept and nourished in the common oviduct of the female. Once it becomes mature larva of the 3rd instar, which takes about a week, it is deposited usually in the shelter of the host. In case of some Hippoboscidae it is dropped freely to the ground or in case of the sheep related *Melophagus ovinus* it is attached to the fleece. Within few hours it turns into pupa. In streblids *Trichobius* spp. this molting takes place within the female. Maturation of pupa takes a month or more. Adult Hippoboscoidea live for several months and a female can produce five to 15 offspring in a lifetime.

Depending on the presence of wings the dispersal of Hippoboscoidea can be active or passive. Some Hippoboscidae are known to carry other ectoparasitic mites and lice on their body (Macchioni et al. 2005).

No studies dealing with population genetics of Hippoboscoidea are available.

Phthiraptera

The order Phthiraptera is species rich and differentiated, containing about 550 species of sucking lice classified in 50 genera and 15 families and approximately 4500 species of chewing lice in 205 genera and 11 families. They are distributed through the whole world on variety of host among birds and mammals.

The evolutionary origin of lice was revealed in relatives of the psocopteran family Liposcelidae (e.g. Lyal 1985). This family contains inquiline species living in nests of endothermic vertebrates. Therefore it is believed that the parasitic strategy of lice originated from such life style. It is also possible that parasitism evolved twice within Phthiraptera, in suborder Amblycera containing part of chewing lice and in the rest of taxa comprising the rest of chewing lice (Ischnocera) and blood sucking taxa (Rhynchophthirina and Anoplura) (e.g. Yoshizawa & Johnson 2010).

The lice have highly and uniquely modified mouth parts serving to feeding on epidermal derivatives in chewing lice and blood in sucking lice. They possess strong short legs with strong claws easing the attachment to the host body. Other adaptations for parasitism are the lack of wings and the reduction of eyes or antennae.

The lice spent their whole life, including the egg, on the host body. The eggs are usually cemented to the base of hairs or feathers of the host. The number of larval instars is reduced to three in order to avoid vulnerability during molting. In optimal conditions the lice can reach up to 12 generations a year. The ability to survive off host varies from few hours in some sucking lice to about two weeks in chewing lice.

Direct contact of host individuals is the primary dispersal mechanism. Quite a common mechanism in Ischnocera but rare in other taxa is phoresy using larger winged blood-sucking insects such as Diptera: Hippoboscidae or Muscidae. Active dispersal via the ground has been suggested for lice as well (Johnson et al. 2012).

The lice exhibit rather high degree of host specificity being mostly monoxenous or oligoxenous (e.g. Zuo et al. 2011). They are often specialized to particular body part of the host. For this reason up to 12 species of lice can be found on one host.

Lice are frequent model organisms in studies dealing with general questions of evolution of parasitism. Since the lice are expected to exhibit large host fidelity they are often studied with regard to their coevolution or cospeciation with hosts. Some studies have shown the evolution of lice and their hosts to be congruent (Demastes & Hafner 1993, Hafner et al. 1994), while some have not (Johnson et al. 2002a, 2002b). During such studies the lice have been shown to exhibit higher rates of molecular evolution than the hosts (Hafner et al. 1994, Johnson et al. 2003).

The phylogeny of lice can also tell about the history of humans (Kittler et al. 2003, Reed et al. 2004). Long history of research on the divergence of human body and head lice is reviewed by Veracx & Raoult (2012) concluding that there are three major clades of head louse and one of them can produce body lice that differ only in phenotype.

Quite a limited number of studies are available on population structure of lice on other hosts. Johnson et al. (2002c) found the genetic structure according to geography to be above structure according the host association in the genus *Columbicola* while both factors shaped the structure in the genus *Psysconelloides*. Whiteman (2006) compared the genetic structure of three lice species parasitizing the Galápagos hawk and found it consistent with their different dispersal modes and other ecological aspects.

Siphonaptera

The order Siphonaptera (fleas) comprise about 2500 described species of obligate ectoparasites classified in 220 genera and 15 families. The position of Siphonaptera among other holometabolous orders was a matter for a broad discussion. Their sister relationship to mecopteran family Boreidae appears to be the most likely option based both on morphology and molecular data (reviewed by Whiting et al. 2008). Such assessment was complicated by the largely modified morphology of fleas, which, together with the inability to polarize characters due to unknown sister group, was also constrain in reconstruction of the in-group phylogeny.

Except for females of the basal family Tungidae, the appearance of fleas is extraordinarily consistent. They are laterally flattened, wingless, they lack compound eyes and their mouthparts are largely modified to sucking blood. They posses ctenidia on body segmernts and their legs are adapted for jumping.

The degree of physical association of adult fleas to their host is very diverse. Some, like *Echidnophaga* spp. or *Tunga penetrans*, are permanently attached to their host by their mouth parts or beneath its skin respectively. The opposite case are fleas associated with birds which spend most of their life in nests and climb on the host only when they need to feed. The rest of fleas fill the gradient between these two modes. Dispersal of fleas on longer distances is mostly passive (e.g. Kuznetsov & Matrosov 2003).

The female produce up to 20 eggs a day and up to several hundreds during her lifetime which can last a year or more. The larvae are apodous and live in the nest of the host feeding on organic material or feces of adults. Most of species have three larval instars, some two. The development can be fulfilled in as little as four weeks including a week spent in pupal stage. The larval development but particularly the pupal stage can be prolonged to a year or more allowing the fleas survive periods without blood supply. The adults of some species are also able to survive over a year without feeding.

Majority of fleas are associated with mammals, only 6% of species comprised in two families are parasites of birds (Marshall 1976). Fleas are rarely monoxenous at species level but higher taxa are usually associated with particular taxa of hosts. About 74% of species are found on rodents, 5% on bats. A lot of information on host specificity of particular flea species is available for broader syntheses such as by Poulin

et al. (2009) who suggested irreversible evolutionary trend towards narrow specialization.

On the other hand, studies on population genetics of fleas are rather scarce. The studies by de la Cruz & Whiting (2003) and Luchetti et al. (2007) deal with mtDNA and in case of the latter study also with nuclear DNA diversity of two of the main human pests *Pulex simulans* and *Tunga penetrans* respectively. Brinkerhoff et al. (2011) found that the population structure of *Oropsylla hirsute* well reflects the population dynamics of its host, prairie dog, shaped by plague that the flea transmits.

Heteroptera

The adaptation of mouthparts to blood feeding in Phthiraptera was prerequisites already in the modifications in primitive Paraneoptera now represented by Psocoptera. These modifications included stylet like laciniae and development of cibarial pump allowing sucking of fluids. The sucking mouth parts in Hemiptera likely evolved independently from those in Phthiraptera. Most taxa of Hemiptera are phytophagous, only in Heteroptera predatory or parasitic groups are found.

Blood feeding evolved at least twice within Heteroptera: Cimicomorpha. First, it happened within probably the most ancestral group of the suborder, the family Reduviidae, likely as a result of association to the habitat of mammals' nests.

Cimicidae and Polycetenidae, usually considered sister groups, were suggested acquire the ectoparasitic lifestyle for similar reasons. They likely evolved within Anthocoridae s. lat. (Schuh & Štys 1991, Tian et al. 2008). Most anthocorids s. lat. live predatorily, but some are inquiline in birds nests (Jung et al. 2010) and some are even facultative blood feeders.

However, the original idea of sister relationship of Cimicidae and Polycetenidae came rather from their common parasitic life style and relation to bats. The family Curaliidae which was described as late as in 2008 (Schuh et al. 2008) was placed sister to Polycetenidae according to recent analysis (Schuh et al. 2009). It is possible that the ectoparasitism evolved independently in Cimicidae and Polycetenidae.

Another feature common not only to Cimicidae and Polycetenidae but to whole Cimicoidea is traumatic insemination. This unusual behaviour most likely evolved only once within the superfamily (Sunghoon Jung pers. comm.). However, the

morphological modifications connected with traumatic insemination have gone through independent evolution in each group.

Heteroptera: Reduviidae: Triatominae

The subfamily Triatominae belongs among 23 subfamilies of Reduviidae, a family basal to Cimicomorpha (Schuh et al. 2009). It contains about 140 described species classified in about 17 genera and five tribes (Schofield et al. 1999). Most species live in Neotropic region, only few species stretch to North America and 13 species are found in India and South-East Asia. One species, *Triatoma rubrofasciata* is considered tropicopolitan.

The species of the subfamily feed on blood of diverse vertebrates, including reptiles. Some authors (e.g. Schofield & Galvao 2009 and other previous works by Schofield) believed that the haematophagy evolved independently in several different lineages of Reduviidae and the subfamily Triatominae only gathers these blood-feeding species. Recent analyses recovered Triatominae as a monophyletic group (Weirauch & Munro 2009, Patterson & Gaunt 2010) or at least as a taxon paraphyletic with respect to genus *Opisthadicus* (Hwang & Weirauch 2012). The relation of Triatominae to *Opisthadicus* in fact only confirmed the idea of inquilinism in mammal nests as their original life style. Their sister relation to genus *Zelurus* suggested in this study documents the New-World origin of Triatominae. The Old-World species were not included in studies by Weirauch & Munro (2009) or Patterson & Gaunt (2010) but their close relation to the rest of Triatominae and their recent spread from America is suggested by Hypsa et al. (2002).

Triatominae physically resemble their predatory relatives among Reduviidae, being mostly above 20 mm long. Their head is characteristically elongated and in contrast to predatory Reduviidae, their rostrum is rather thin, straight and if not used it is placed ventrally parallel to the body.

They inhabit shelters of their hosts and climb on their hosts only when they need to feed or disperse. Their development goes from egg through five larval instars which all, except for the first one, need more than one blood meal in order to molt to another. The life cycle can be completed in three to four months but it usually takes more than a year. During the lifetime the female can lay up to 1000 eggs but usually not more than 200.

As winged, Triatominae are able to disperse actively. *Triatoma infestans*, though, was shown not to fly in altitudes over 2750 m asl (Richer et al. 2007). However, passive phoretic dispersal by their hosts is likely the leading mechanism (Schofield et al. 1999).

Their host choice is shaped by their habitat and available sources rather than by host preferences (Rabinovich et al. 2011). About 20 species are known as vectors of *Trypanosoma cruzi*, the organism responsible for the Chagas disease, among humans. Such species often establish domestic populations with more or less contact with the wild sylvatic forms. The domestic forms then, beside humans, use variety of domestic or synanthropic animals.

This way the populations of triatomids are inconsistent and setting species limits often shows problematic. A lot of studies dealing with phylogeny and biogeography on lower taxonomic levels are available (e.g. Marcilla et al. 2002, Monteiro et al. 2004, Pfeiler et al. 2006). The studies use diverse molecular markers whose relevance is reviewed and discussed by Mas-Coma & Bargues (2009).

One of the most studied species is *Triatoma infestans* which, together with *Rhodnius prolixus*, is the most important vector of the Chagas disease. Revealing the phylogeny, biogeography and population structure of the two species is also aimed to set better control. The only original wild sylvatic population of *T. infestans* was found in Bolivian Andes infesting mainly guinea pigs (Bargues et al. 2006). Guinea pigs were domesticated by humans and following them *T. infestans* spread over whole Latin America. Two basic lineages of the dispersal are distinguished: Andean one in Bolivia and Peru and low-land in the rest of the countries. Both these lineages were found in Peru among humans but also setting secondary sylvatic populations (Torres-Perez et al. 2011). Based on analysis of microsatellites the sylvatic populations were shown to be able to actively re-colonize insecticide treated houses (Richer et al. 2007). Another microsatellite analysis (Marcet et al. 2008) showed that the dispersal of *T. infestans* is female-biased.

Heteroptera: Polytentidae

The family comprises about 30 species classified in five genera and two subfamilies distributed in tropics of both Old and New World. Members of the family possess several significant morphological features connected with their parasitic life style. Their body is

dorso-ventrally flattened and they lack eyes. They possess ctenidia, similarly as fleas. Their wings are neotenic and appear as non-articulated mesonotal lobes.

Similarly as in Cimicidae the female is mated into a specialized integumental organ – the ectospermaldege - from where the sperm migrates to the mesodermal mesospermaldege. These organs are located at the right metacoxal articulation membrane and the male mates the 3rd instar larva of a female.

Polyctenidae are permanently attached to their bat host. Their life cycle is adapted for this attachment through viviparity: the first larval instars are nourished within the female by pseudoplacenta and only three post natal instars feed on the host.

Any dispersal of Polyctenidae bugs is only by contact of host individuals. This is one of the reasons why the species of Polyctenidae are usually considered mono- or oligoxenous (Marshall 1980). However, this assumption is based on limited number of records for each species. According to data by Maa (1964) it seems the host range does not exceed a genus of bats. A recent experiment showed that Polyctenidae can readily accept a non-specific host (Dick et al. 2009). However, this is single such evidence available and the original and novel hosts were also congeneric.

No studies dealing with population genetics of Polyctenidae are available.

Heteroptera: Cimicidae

The family Cimicidae includes about 110 species classified in 24 genera and six subfamilies (Henry 2009). The representatives are distributed on all continents, only Australia lacks autochthonous species (Usinger 1966).

Cimicids are blood-sucking parasites which use the body of their host usually only when they need to feed. The rest of the time they spend in refugia in the shelter of the host, often aggregated into clusters according to their sex and age.

Before a cimicid become an adult, it undergoes egg and five larval stages. Each larval instar needs at least one blood meal to pass to another. In optimal conditions the adults feed every three to seven days depending on temperature and instar. Female of the bed bug, *Cimex lectularius* Linnaeus, 1758 was shown to lay around 5 eggs per week for a period up to 18 weeks (Kemper 1936). In conditions of temperate climate the source of blood in wild hosts is available only during the breeding season and the cimicids has to starve for long months. The adult bed bug was shown to be able to starve up to 18 months in temperatures around 13°C and eight months in temperatures

above 18°C. The early instars endure much less, thus the host finds mainly adults and late instars when it comes back after winter.

In cimicids, male inseminate the female inserting a modified asymmetric left paramere into paragenital opening and injecting sperm into ectospermaledge (Carayon 1966) located in membrane between abdominal segments (different position in different taxa). Similarly as in Polyctenidae, the spermatozoa migrates to mesospermaledge where aggregate and activate due to the action of male „mesadenal" fluid (Davis 1966, Rao 1972). Once activated, the spermatozoa move through the haemolymph along the oxygen gradient and enter the seminal conceptacles. These female structures store and nourish the spermatozoa similarly as non-homologous normal insect sperm storage organs (bursa copulatrix and spermatheca). The sperm can be stored up to seven weeks in the bed bug *Cimex lectularius* (Mellanby 1935, Davis 1966) or even up to whole winter as shown in the American swallow bug *Cimex* (formerly *Oeciacus*) *vicarius* (Horváth, 1914) (Loye 1985). It was not tested in other species or the population of the bed bug on bats. The ability for such a long storage may be usual among cimicids using migratory hosts and it is possible that it just has been lost in the bed bugs living with people who usually constitute a stable blood source.

In cimicids, several interesting behavioral features have been suggested to have developed as consequences of traumatic insemination and the resulting conflict between sexes. Despite the presence of structures developed to ease traumatic insemination, it is still harmful for female which tries to avoid excess mating frequency (Stutt & Siva-Jothy 2001). In *C. lectularius*, it was shown that males prefer to mate freshly fed females which are less physically able to resist but also represent better prospective reproduction mates (Reinhardt et al. 2008). Males use less than 20% of the amount of sperm available in order to be able of multiple mating (Reinhardt et al. 2011). As a counteraction, females of *Afro cimex constrictus* Ferris and Usinger, 1957 are polymorphic: some mimic male appearance likely in order to reduce the frequency of costly mating (Reinhardt et al. 2007).

The host choice of the family as the whole is rather narrow compared to other blood feeding insect groups. Bats are very likely the original or ancestral host of the family, as the majority of higher taxa is related to them (Horváth 1913), mostly to families Vespertilionidae and Molossidae (Marshall 1982). Usinger (1966) suggested that cimicids have switched from bats to birds as exclusive hosts on four occasions: the whole subfamily Haemosiphoninae, the genus *Paracimex* Kiritschenko, 1913, the

former genus *Oeciacus* Stål, 1873 and the species *Cimex columbarius* (Jenyns, 1839). The last three taxa are from the subfamily Cimicinae.

The bats share several ecological features with the hosts among birds. These are mainly from families Hirundinidae and Apodiidae. Similarly as bats, they are colonial and breed in spaces isolated from the outside conditions. Furthermore, their nests are often located in caves or buildings, which are frequent shelters for bats as well. This eases the contact between these groups of hosts and, as a result, alternative host encounter possibly resulting in host switch. Actually, bats are known to use nests of birds of the family Hirundinidae (Schulz 1995, Loye & Carrol 1998, Ritzi et al. 2001) or even to breed there (Kazuhiko Hirata pers. comm.).

The host specificity of particular species cannot be generalized for the family. For many species only few records exist, if not only the type material. Therefore, only species from which a considerable material exists are mentioned below.

Few cimicids species can be described as monoxenous. *Primicimex cavernis* Barber, 1941 is believed to live only on the bat *Tadarida mexicana brasiliensis* (Ueshima 1968), *Camincimex furnarii* (Cordero and Vogelsang, 1928) on the oven bird *Furnarius rufus*, *Psitticimex uritui* Lent and Abalos, 1946 on the parrot *Myiopsitta monacha cotorra* and *Cimexopsis nyctalis* List, 1925 on the chimney swift *Chaetura pelagica* (Usinger 1966).

Some species can be regarded as having one principal host and being occasionally recorded on others. It is *Pipistrellus kuhlii* for *Cacodmus vicinus* Horváth, 1934 (reviewed by Quetglas et al. 2012), *Delichon urbica* for *Cimex* (formerly *Oeciacus*) *hirundinis* Jenyns, 1839 (e.g. Országh et al. 1990, Trilar et al. 1997) or *Petrochelidon pyrrhonota* for *C. vicarius* (Eads et al. 1980, Loye 1985).

Species of the genus *Cimex* are usually known from a variety of bat species of the family Vespertilionidae. The host ranges for the *Cimex pipistrelli* (Jenyns, 1839) group and *C. lectularius* are reviewed in detail by Balvin et al. (2013c). Lack of reports from some bat species and the limited overlap of species recorded for the *C. pipistrelli* group and *C. lectularius*, as well as results of transplant experiments (Růžičková & Bartonička 2011), suggest that not all Vespertilionidae species in the region are suitable hosts. Similarly broad host range of bat species within Vespertilionidae is reported for *Cacodmus villosus* Stål, 1855, *C. sumatrensis* Ferris and Usinger, 1957 or *Aphrania elongata* Usinger, 1966 (Usinger 1966).

Beside the bed bugs *Cimex lectularius* and *C. hemipterus* parasitizing bats and man there are only few cimicids whose host ranges comprise more than one family. The host range of *Haemosiphon indorus* Dugès, 1892 comprises five bird families including birds of prey nesting outside (Usinger 1966). The species is an important pest of poultry. *Stricticimex antennatus* Ferris and Usinger, 1957 was observed to feed on bats of three families (Overal & Wingate 1976): beside Vespertilionidae they are Nycteridae and Rhinolophidae that do not occur among hosts of other cimicids. However, the observation comes from a single cave inhabited by bats of all the three families. Therefore, it is not possible to state whether all of them can serve as the primary host at other locations.

By a recent experiment, Wawrocka & Bartonicka (2013) found that samples of the bed bug *C. lectularius* collected from humans or bats fed less frequently and had higher mortality on the non-native host. This suggests a need for a large extent of adaptation to particular hosts in a cimicid formerly considered generalist but also illustrates the deep divergence between the populations on bats and humans.

Cimex lectularius was shown to actively disperse from refugia after certain time of starving at certain temperature; females were dispersing more actively (Mellanby 1935, Johnson 1941). Pfister et al. (2009) showed that the active dispersal of bed bugs depends on the population structure and abundance and confirmed that females spread first. *Cimex vicarius* was shown to be able to walk up to 3 meters among individual nests of swallows (Brown & Brown 1996). However, cimicids mostly rely on passive transport. *C. vicarius* has been observed sitting at the rim of the nests in spring (Loye 1985). Birds coming after winter first check the parasite load of nests before nesting. The bugs readily attach to their feathers and let the birds carry them to other nests or colonies. Similarly, the intentionality of attachment to the body of the host in order to disperse was suggested in bat related species of the genus *Cimex* (Heise 1988). The population structure and N_e are then largely determined by the spatial isolation of the host shelters and the contacts between host species within the host range.

No phylogenetic analysis of the family based on molecular data is available. The life style and distribution of cimicids do not allow consistent targeted sampling. However, coincidental findings of diverse taxa are increasing and the material needed is being slowly accumulated. Phylogenetic hypothesis based on morphology has been attempted by Usinger (1966) who admits it is far from being resolved. It mainly relies

on the morphology of genitalia. Their ancestral state for the family is not known, also because the sister taxon is uncertain.

Except for the research shown in the present thesis, only few studies dealing with population genetics of cimicids are available, all regarding the bed bug *C. lectularius*. Based on mitochondrial 16S ribosomal gene, Szalanski et al. (2008) revealed surprisingly high diversity of the population of the bed bug on humans in the USA suggesting that no bottleneck due to DDT occurred after 2nd World War or substantial reservoirs among other hosts. The microsatellite markers have been developed by Booth et al. (2012) who commented on the population dynamics within high-rise buildings supporting both active and passive dispersal. Their subsequent study (Saenz et al. 2012) revealed low diversity within particular infestations but high diversity in the population across the USA exhibiting no structure according to geographic distances. Their data suggested that the infestations are mostly founded by few individuals or rather a single female and basically confirmed the conclusions of Szalanski et al. (2008) suggesting multiple sources of the current bed bug spread.

Aims of the thesis and methods used

1. Solution of the taxonomy of the West-Palaeartic species of the *Cimex pipistrelli* group was approached using detailed revision of diagnostic morphological characters and sequences of both mitochondrial and nuclear genes. Examination of the population structure and geography as well as the host-associated phenotypic variation was performed using the same molecular and morphological data.
2. Examination of the population structure and phenotypic variation in the bed bug *Cimex lectularius* was done using sequences of mitochondrial genes, screening of microsatellite loci and thorough morphological analysis. The data obtained from samples of populations from bats and people were used to suggest the history of host association of the bed bug and express the current relationship of the two populations.
3. The phylogeny of the species of the genera *Cimex* and *Oeciacus* with respect to switches in host association was studied using sequences of two mitochondrial and three nuclear genes. The synonymy of the genera *Cimex* and *Oeciacus* was suggested and discussed using revision of diagnostic morphological characters.
4. A considerable amount of ecological and biogeographical information was drawn from the data on the collection of material used for the population genetic and phylogenetic studies.

Factors of diversification in the genus *Cimex* and the family Cimicidae: comparison with other ectoparasitic taxa and contribution based on the results of the thesis

The precondition of emergence of ectoparasitism in Cimicidae, together with Polycytenidae, is not exceptional among other ectoparasitic groups. In fact in all parasitic Paraneoptera the parasitism was suggested to have developed from inquilinism in vertebrate nests. This course of evolution is likely the shaping factor for the host choice in Cimicidae (and maybe in Polycytenidae). It comprises animals living almost only in nests or roosts closed from the outside environment which seems to be unfavorable for cimicids.

Unlike Polycytenidae, cimicids adopted the host exploitation strategy inhabiting the shelter of host and using the host only for feeding and dispersal. This behavior defines the deme structure of cimicids species, i.e. the units of freely interbreeding individuals. In ectoparasites closely attached to the body of host, such as Polycytenidae or Phthiraptera, the deme is represented by the infrapopulation of individuals on a single host individual. In cimicids associated with bats or single living birds the deme is represented by the bugs living in a single bat roosts or a bird nest. In species parasitizing colonial birds the deme structure is not that clear being shaped both by the whole colony and individual nests. The term infrapopulation is not applicable in cimicids.

The life strategy adopted by cimicids constitutes an advantage among other ectoparasites: cimicids do not have to compete for the space on the host body (Marshall 1982). Such competition has been suggested to occur between Polycytenidae, Nycteribiidae and Streblidae resulting in exclusive presence of either of the groups on a bat species (Wenzel & Tipton 1966).

Obviously, in comparison with ectoparasites closely attached to the host body the cimicids have much higher ability to survive off the host body. While it is only few hours for some fleas or Polycytenidae, cimicids has to be able to survive starving for months when the host is absent for overwintering.

Regarding the survival outside the shelter of the host, cimicids were shown to be able to deal with the outside conditions for a limited amount of time and disperse actively for short distances. *Cimex vicarius* was shown to walk among swallow nests within a colony. *Cimex hirundinis* which usually inhabits nests of synanthropic birds is

often found on the outside walls (Országh et al. 1990, Antonín Reiter pers. comm. regarding the sample IC 893 listed in Table 1 in Balvín et al. 2013b) and reported to enter homes probably through windows (Gogala & Gogala 1986, Petr Lelek pers. comm.), similarly as *C. vicarius* (Eads et al. 1980).

Bat associated species living in stable colonies of philopatric bats usually sit tight in the roosts all year, faithfully waiting for bats to come next spring. *Cimex lectularius* collected from bat roost in winter even refused to feed in captivity until spring (Tomáš Bartonička pers. comm.). Problems with cimicids penetrating homes are reported only from bat species using temporary summer roosts, such as *Nyctalus noctula*. In such cases the bat bugs can also use outside walls to enter the building (mr. Hora pers. comm. regarding the sample IC 185 listed in Table 2 in Balvín et al. 2013c).

The bed bug *Cimex lectularius* is also able to disperse this way among apartment units (Lýsek 1966). According to our morphological analyses (Balvín et al. 2012a, Balvín unpubl.) the bed bugs from humans have longer legs in comparison to the population on bats. Such legs might have developed for the purpose of active dispersal among people homes within a building or settlement. Such dispersal is usually not possible among individual bat roosts scattered over the country.

Anyway, the passive dispersal using the host is definitely the leading mechanism in spread of cimicids. They spend most time off host on which they appear when they need to feed on its blood. The probability of leaving the shelter of the host on such occasion is quite low. The prevalence of adults and especially females found on bats outside roosts suggests that cimicids increase this probability by intentional attachment to the host body in order to disperse to new locations (Heise 1988, Balvín et al. 2012b). Even alone, mated females are prospective founders of new infestations. This view is also supported by experimental studies on the bed bug (Mellanby 1935, Johnson 1941, Pfiester et al. 2009) suggesting females leave aggregations earlier due to either hunger or high abundance. Such female biased dispersal is also found at least in Triatominae (Marcet et al. 2008) and is likely prerequisites by the ability to store sperm in female genitalia.

As suggested by Balvín et al. (2012b) based on data on cimicids found on mist-netted bats the frequency of the dispersal can largely differ among different host species of a single cimicid species possibly according to the ecology of the hosts. Based on analysis of microsatellites (Booth et al. manuscript) the diversity of *C. lectularius* in bat roosts is considerably higher than of those in human dwellings. It obviously reflects the

fact that the infestations of humans are found by limited number of females and have little chance to migrate among other infestations before they are exterminated. However, at the same time this difference documents the actual migration among roosts of the host bat species, i.e. mainly *Myotis myotis* in the case of the bed bug. Unfortunately the rate of migration cannot be compared with other bat or cimicids species. The microsatellites suggested no further structuring of the population of the bed bug on bats. This is rather surprising since most samples come from *M. myotis* which is a philopatric bat species with low migration (Horáček 1985, Berková et al. 2013) and low frequency of transmission of cimcids (Balvín et al. 2012b).

Anyway, the overall dispersal rate in Cimicidae is likely much lower than in ectoparasites closely attached to the host body. This restricts the maintenance of the genetic diversity which is necessary for keeping locally adapted. Possibly frequent bottlenecks due to dying off when overwintering in breeding roosts or nests of the hosts also lower the genetic diversity in demes. It is possible that the multiple mating of cimicids serves as compensation. It has been shown that progeny of a single female can have multiple fathers (Stutt & Siva-Jothy 2001). However, the sperm of the second male to mate the female is more successful. This male recognizes that the female is not virgin and reduces the volume of ejaculation in order to save it for other females (Siva-Jothy & Stutt 2003).

The degree of host specificity is rarely possible to be generalized for the whole parasitic taxon and thus not possible to be compared among different taxa. It is definitely very diverse in Cimicidae. The results gathered during the course of research presented in the thesis documents the host specificity of the studied taxa on several occasions.

The switch of the bed bug from bats to humans and possibly repeated switches between birds and bats by species within the genus *Cimex* represent major changes in host choice. In both cases they have been accompanied by large adaptive change in phenotype and genetic differentiation. The switch from bats to birds resulted in emergence of the species of the former genus *Oeciacus* (Balvín et al. 2013b). Similarly, in the bed bug the switch from bats to different host group – humans – was accompanied by large divergence in mitochondrial and nuclear DNA and phenotype (Balvín et al. 2012a, Booth et al. manuscript). The divergence resulted in limited ability to survive on non-specific host (Wawrocka & Bartonička 2013). The analysis of microsatellites suggested that there is no recent gene flow between the populations of

the bed bug on bats and on humans. The population on humans can be thus regarded an emerging new species. Both situations document that cimicids species are not generalists enough to be able to exploit such distant host groups. Unfortunately, *C. columbarius* which is likely another example of such situation possibly being another daughter species of *C. lectularius* was not sampled.

The morphological differentiation of the bat bugs of the *C. pipistrelli* group among different hosts documents the need for adaptation to different host species within the usual host range (Balvín et al. 2013a). It was not possible to distinguish if the morphological differentiation stems from local adaptation or is caused by phenotypic plasticity.

Based on an extended sample of *C. lectularius* counting, beside samples from humans and other bat species, 154 specimens from 29 roosts of *Myotis myotis* and 41 specimens from 8 roosts of *M. emarginatus* measured in 40 characters no morphological differentiation was found among bed bugs from the two bat species (Fig. 1, Balvín unpubl.). These two host bat species are ecologically similar. In the *C. pipistrelli* group the differentiation was shown between bat species with quite different ecology. It is possible that the need for adaptation to particular bat species stems rather from the ecology of the host than its physical qualities. First, it can be the character of roosts inhabited by particular bat species. Second, it can be the degree of philopatry of the bat colonies: the phenotype of the cimicids on the migratory crevice dwelling bats would be shaped by the need of frequent dispersal among multiple roosts or need for ability of temporary starvations during the breeding season in contrast to cimicids on non-dwelling bats which are faithful to their roosts.

However, the smallest specimens of both *C. lectularius* and the *C. pipistrelli* group were always found on *Pipistrellus* spp. which are far the smallest bat species in both host ranges. Therefore it is likely that the character of the body of the host shapes the body of the parasite anyway.

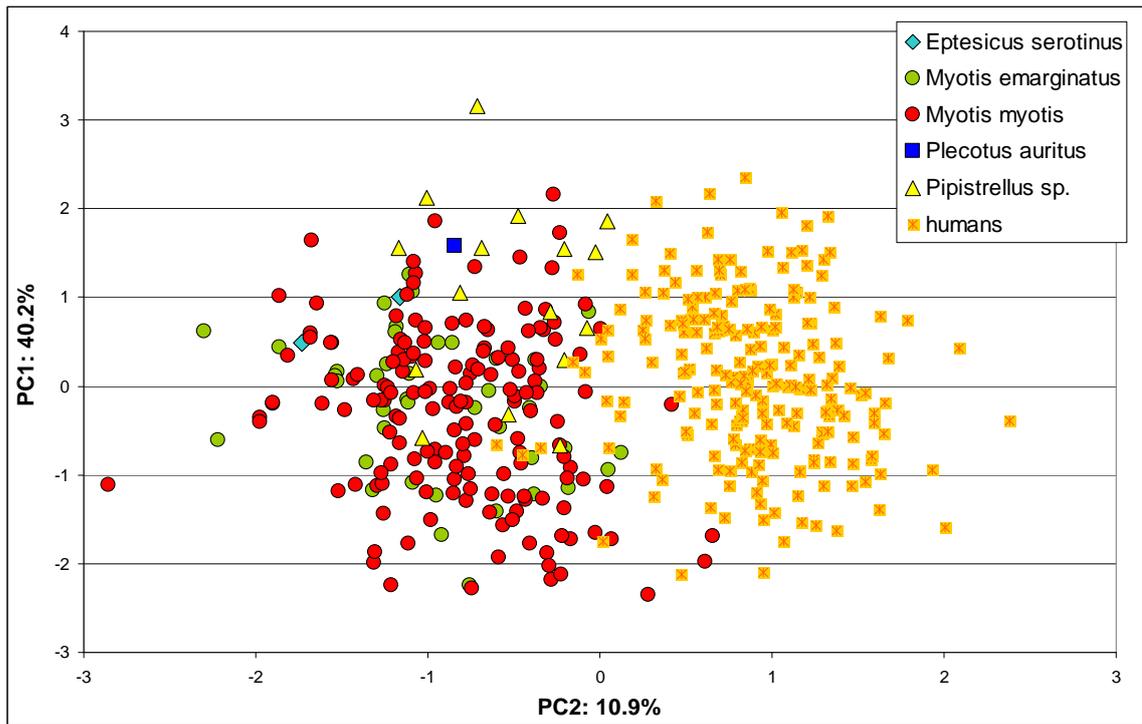


Fig. 1. PCA based on 40 morphometric characters measured in 445 specimens of *C. lectularius*. Post hoc test in ANOVA of factor scores on PC1 and PC2 did not show any difference between bed bugs from *Myotis myotis* and *M. emarginatus* (Balvín unpubl.).

Conclusions

The data included in the thesis documented several diversification events in taxa of the genus *Cimex*.

1. The former genus *Oeciacus* diverged within the genus *Cimex* due to host switch from bats to birds of the family Hirundinidae (Balvín et al. 2013b). The switch has been accompanied by a large change in phenotype which made the earlier researchers classify the bird associated species in a separate genus. Moreover, it is possible that each of the species associated with Hirundinidae has evolved by an independent switch from bats to birds and the change in phenotype was convergent in these species. The monophyly of bird associated species of the genus *Cimex* was suggested by nuclear molecular data while their polyphyly or possibly the paraphyly by mitochondrial data.

2. A new species of *Cimex* associated with Hirundinidae was discovered in a population collected in Japan (Balvín et al. 2013b). Similarly as *Cimex hirundinis* has a principal host in *Delichon urbica* and *C. vicarius* in *Petrochelidon pyrrhonota*, this species seem to be associated mainly with the Asian house martin, *Delichon dasypus*. As any samples from *D. dasypus* from continental Asia were not available in order to determine which species they belong to, it is not sure if this species evolved by a host switch or by isolation on Japanese islands.

3. The divergence of the bed bug *Cimex lectularius* according to the association to bats and people showed deep both in genotype and phenotype (Balvín et al. 2012a, Booth et al. manuscript, Balvín unpubl.). As we did not detect any current gene flow between the two populations, the degree of divergence in the bed bug goes beyond the definition of host races sensu Dres & Mallet (2002). Therefore, the population of the bed bug on humans can be regarded as an organism undergoing incipient speciation.

4. The West-Palearctic *Cimex pipistrelli* group contains two distinct mitochondrial haplogroups (Balvín et al. 2013a). We found no evidence that these haplogroups represent separate species. If they do not, one of these haplogroups, likely the more conservative one has probably a different geographic origin and recently spread through Europe. However, previous hybridization experiments suggested the existence of at least two species of the *C. pipistrelli* group in Europe which may correspond to the haplogroups. Anyway, the haplogroups do not correspond to species defined by morphology in the previous taxonomic solutions.

5. The host associated morphological differentiation in the *C. pipistrelli* group points to a need of adaptation to different host species of bats (Balvín et al. 2013a). However, based on the study of only mitochondrial genes along the morphology we did not determine if the differentiation is a result of local adaptation due to selection or is caused by phenotypic plasticity.

Beside the population genetic and phylogeographic data on the taxa of the genus *Cimex*, the research presented in the thesis has documented several ecological features of the studied organisms. Balvín et al. (2012b) discuss the intentionality and diversity in the frequency of passive transport of cimicids by different bat species. On mist-netted bats only adult and mainly female cimicids are found, though the larvae can prevail in the roosts. The females, if mated, are able to colonize a new locality by themselves. Therefore it is likely that they tend to disperse more often than males which need a female to reproduce or larvae which less likely survive to become adult and mate in a new location. The frequency of transmission of cimicids differs among bat species possibly due to response of cimicids to their different ecology.

In congruence with the situation in the USA (Saenz et al. 2012), the population of the bed bug on humans in Europe was shown to lack spatial population structuring (Booth et al. manuscript). The reason lies likely in the increased movement of people and goods such as furniture on both continents which was also likely one of the causes for the recent resurgence of the bed bug. The diversity across the continents suggests multiple sources of the resurgence. The population on bats was significantly more diverse within individual infestations than the population on people due to the stability of the bat colonies and multiple introductions of bed bugs in the former and the pest control in human dwellings in the latter.

Another support for the depth of the differentiation of the bed bug between bat and human hosts was found in the different pattern of mutations in the α -subunit of the gene for voltage-gated sodium channel responsible for the resistance to pyrethroids (Booth et al. manuscript). The population on bats consistently exhibited the “natural” haplotype. The European population on humans mostly appeared mutated but the pattern was found different from that in the USA suggesting that neither of the populations has been a recent major source for the other.

The finding of a lack of recent contact between the population of the bed bug on bats and people is important both from the points of view of the bed bug control and the bat protection. Though the bed bug often inhabits roosts of synanthropic bat species

these infestations are almost no risk for humans. Though the protection of bats in buildings is regulated by law, in fact it rather depends on the will of the owners. If the bats were broadly known to transmit bed bugs, the law enforcement would be more complicated.

The host range among bats was extended for both *Cimex lectularius* and the *C. pipistrelli* group (Balvín et al. 2012b, 2013c). The *C. pipistrelli* group was found common in roosts of many species in Central Europe, while *C. lectularius* can be regarded common only in roosts of *Myotis myotis* and *M. emarginatus* in the region. The geographic distribution of the *C. pipistrelli* group and *C. lectularius* on bats has been reviewed and extended by several countries.

Future perspectives

On the course of any scientific research usually more questions are raised than answered. The research presented in the thesis reached several rather surprising results contradicting the traditional concepts not only of taxonomy but also of basic genetics.

The taxonomy of West-Palaeartic species of the *C. pipistrelli* group was not entirely solved based on mtDNA. Variable nuclear markers have to be used in order to disentangle the species limits. The microsatellite markers are just being developed by Tomáš Bartonička (Faculty of Science, Masaryk University, Brno, Czech Republic). Such markers will allow studying the population structure in more detail than mtDNA. They can describe the differentiation and gene flow among bugs from different host bat species and thus tell if the host-associated morphological differentiation and the probable behavioral variation are due to local adaptation or plasticity.

The finding that the bird associated species of the former genus *Oeciacus* evolved, possibly independently, within the genus *Cimex* opens a field for a broad phylogeographic study on taxa from the whole Palaeartic region with respect to changes in host association.

In the population genetic study of the bed bug *Cimex lectularius* only about one third of the gathered material from humans was screened for microsatellite loci. The rest will be screened soon, the data joined with information on the population in the USA and used for an unprecedentedly detailed global study aiming to explain the reasons and mechanisms of the current bed bug spread.

The discovery of common heteroplasmy likely due to paternal inheritance in the bed bug opens an attractive field of research on essential and indeed general questions of inheritance of genetic material.

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