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**Department of Zoology**  
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# **Behavioural and genetic study of premating isolation in the house mouse hybrid zone**

**Ph.D. Thesis**

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*September 16<sup>th</sup> 2008.*

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## PREFACE

Speciation, a continuous process leading to evolution of separate species, which are reproductively isolated from each other, is a central tenet of contemporary evolutionary biology. Hybrid zones, i.e. areas where distinct populations meet, hybridize and exchange genes, present ideal opportunities to get insights into the origins and mechanisms of this amazing evolutionary process. During my studies I have been focusing on behavioural genetics, being interested in how differences in behaviour of different species may contribute to their speciation. I have capitalized on advantages of the mammalian model organism – the house mouse – which, owing to the knowledge of its genome sequence, existence of natural hybrid zones between subspecies and to ease with which it can be kept and bred in captivity, may be seen as a unique evolutionary model to address and solve basic speciation questions.

The primary goal of this thesis was to describe and test a potential role of behavioural isolation, more specifically the divergence of subspecies-specific signals and associated preferences as a prezygotic barrier contributing to the speciation between two house mouse subspecies *Mus musculus musculus* and *M. m. domesticus*. The hypotheses behind the aims listed below have been tested in natural populations as well as using wild-derived inbred strains.

The thesis is based on four scientific papers, hereafter referred to as Supplement 2–5, representing successive solving of partial aims described below. Two of these papers have been published in peer reviewed journals, one was accepted to *Behavioural Processes* and one to *BMC Evolutionary Biology*. A part of the Introduction, concerning the house mouse as a model for genetic and evolutionary studies and summarising the results presented in Supplements 2–5, is based on a review article accepted for publishing in *Acta Musei Moraviae* (Supplement 1).

The aims of the study were as follows:

- Using behavioural (Supplement 2) and molecular (Supplement 5) analyses, to test the role of salivary Androgen binding proteins, proposed to act as a signal leading to assortative mating between both subspecies, in natural populations across a house mouse hybrid zone.
- To derive inbred strains representing both subspecies with sufficient genetic and phenotype variability suitable for analyses of the genetic architecture of traits potentially involved in speciation between both subspecies (Supplement 3).

➤ To describe behavioural components participating in subspecies-specific recognition by assessing the relative signalling potential of candidate subspecies-specific signal compounds and their possible combined effect on assortative preferences of two wild-derived inbred strains representing both subspecies (Supplement 4).

Supplements:

**Bímová, B.** House mouse as a model for genetic and evolutionary studies. *Folia Mendeliana, Scientiae Naturales*, supplementum ad *Acta Musei Moraviae*, in press. (Supplement 1)

**Bímová, B.**, Karn, R.C. and Piálek, J., 2005. The role of salivary androgen binding protein in reproductive isolation between two subspecies of house mouse: *Mus musculus musculus* and *Mus musculus domesticus*. *Biological Journal of the Linnean Society*, 84: 349–361. (Supplement 2)

Piálek, J., Vyskočilová, M., **Bímová, B.**, Havelková, D., Piálková, J., Dufková, P., Bencová, V., Ďureje, Ľ., Albrecht, T., Hauffe, H. C., Macholán, M., Munclinger, P., Storchová, R., Zajícová, A., Holář, V., Gregorová, S. and Forejt, J., 2008. Development of unique house mouse resources suitable for evolutionary studies of speciation. *Journal of Heredity*, 99: 34–44. (Supplement 3)

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Macholán, M., Baird, S.J.E., Munclinger, P., Dufková, P., **Bímová, B.** and Piálek, J. Genetic conflict outweighs heterogametic incompatibility in the mouse hybrid zone? *BMC Evolutionary Biology*, in press. (Supplement 5)

All behavioural analyses have been carried out at the Department of Population Biology, Institute of Vertebrate Biology of the Academy of Sciences of the Czech Republic, Studenec. The material used in my thesis and all manuscripts have resulted from a long-term collaboration on the study of the Czech-Bavarian transect across the house mouse hybrid zone involving the Institute of Animal Physiology and Genetics, ASCR, Brno; Biodiversity Research Group, Department of Zoology, Charles University in Prague; and the Butler University, Indianapolis, USA .

Bímová, B., 2008. Behavioural and genetic study of premating isolation in the house mouse hybrid zone. Ph.D. Thesis. Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Studenec and Department of Zoology, Charles University, Prague. 33 pp. Supplements 1-5.

## ABSTRACT

Behavioural isolation includes all differences in courtship behaviour that allow the recognition and successful mating only within members of the same species and therewith reduce the probability of maladaptive hybridization. It can be an efficient barrier to gene flow between closely related taxa and contribute to or potentially complete their speciation. An ideal opportunity to study the role of behavioural isolation during the speciation process represent closely related or recently diverging taxa, where the exchange of genes still occurs and the process of speciation was not achieved. The house mouse can be seen as a unique model to address speciation genetic questions. This species is a great laboratory animal with described genome sequence in one side and with the natural hybrid zones between different mouse subspecies on the other hand. In my thesis I studied the role of assortative mating leading to behavioural isolation between two subspecies of the house mouse *Mus musculus musculus* and *M. m. domesticus* in both natural populations from the Czech-Bavarian transect across their hybrid zone as well as in the laboratory, using wild-derived inbred strains representing both subspecies.

I found that there is a strong divergence in both the signal and preference parts of the subspecies-specific recognition system between the two subspecies. I analysed the role of salivary Androgen binding proteins (ABP) that have been proposed to act as a signal leading to assortative mating between both subspecies. However, our results of both behavioural and genetic studies did not fully confirm this hypothesis, indicating that ABP are not in itself an efficient barrier to gene flow between both subspecies. ABP may participate on a complex system of subspecies-specific recognition, probably in transmitting the information between interacting animals in close contact. Contrary, long lasting signals such as urine and faeces seems to be more important subspecies-specific indicators and especially the former are proposed to be hot candidates for speciation traits for future studies.

Based on our results we conclude that behavioural isolation in the house mouse hybrid zone may play an important role as a barrier to gene flow and that this barrier is asymmetric between both subspecies, involves both males and females and should be seen as a complex of at least two different strategies: the assortative mating in one side and male aggressiveness in the other. The extent to which both strategies participate on the mouse speciation remains uncertain and needs further analyses mainly of the epi- and genetic architecture of involved behavioural phenotypes. For that purpose we prepared new wild-derived inbred strains representing genome of both subspecies with sufficient genetic variability and differences in studied behavioural traits. These strains represent a unique tool for speciation genetic studies and in combination with possibility to test candidate speciation genes in natural conditions they offer an excellent opportunity to get insights into genetic architecture of a complex behaviour and its role in speciation.

Key words: speciation, behavioural isolation, hybrid zone, salivary Androgen binding proteins, urinary signals, wild-derived inbred strains



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## ABSTRAKT

Etologická izolace zahrnuje veškeré, často ritualizované prvky chování obou pohlaví, které umožňují rozpoznání příslušníků vlastního druhu na úkor ostatních a snižují tak pravděpodobnost nevýhodného mezidruhového křížení. Schopnost rozpoznat příslušníky vlastního druhu a přednostně se s nimi pářit může představovat silnou bariéru toku genů mezi blízce příbuznými druhy a podílet se tak na dokončení jejich speciace. Ideálním prostorem pro studium vzniku a mechanismu působení reprodukčně izolačních bariér představují hybridní zóny, oblasti, kde dochází ke kontaktu částečně izolovaných populací, jejich křížení a vzniku hybridního potomstva. Díky svým vlastnostem ideálního laboratorního organismu, známé sekvenci celého genomu a přítomnosti několika hybridních zón mezi jednotlivými poddruhy, představuje myš domácí ideální modelový organismus pro speciálně genetické studie. Ve své disertační práci jsem se zabývala významem etologické izolace při speciaci dvou poddruhů myši domácí: *Mus musculus musculus* a *M. m. domesticus*. Odlišnost poddruhově-specifických pářících signálů a schopnost výběrového páření byly studovány jak v přírodních populacích podél česko-bavorského transektu napříč hybridní zónou, tak u inbredních kmenů odvozených od divokých populací obou poddruhů.

Slinné Androgen vázící proteiny (ABP) byly navrženy jako signály v poddruhově specifickém rozpoznávání a prezygotické izolaci myši domácích. Výsledky genetických i behaviorálních studií v přírodních populacích z hybridní zóny však tuto hypotézu nepotvrdily. Naznačují, že ABP mohou být pouze součástí komplexního rozpoznávacího systému mezi oběma poddruhy a podílet se spíše na přenosu informace v blízkém kontaktu mezi jedinci. Naopak, močové signály nebo trus, mající schopnost uchovat a vysílat informaci po delší dobu se zdají být významnějšími signály v poddruhově specifickém rozpoznávání a zejména hlavní močové proteiny mohou být horkými kandidáty jako poddruhově specifické indikátory.

Naše výsledky potvrdily, že etologická izolace v hybridní zóně myši domácích je asymetrická mezi oběma poddruhy i pohlavími. Oproti obecně uznávanému předpokladu byli samci ve všech experimentech více vybíraví než samice. Jedním z možných vysvětlení je, že etologická izolace u myši domácích představuje komplex zahrnující dvě odlišné strategie přednostního páření a samčí agresivity. Podíl a význam, který obě strategie mají na výsledné reprodukční izolaci mezi oběma poddruhy však dosud nebyl zcela objasněn a vyžaduje další, důkladnější studie, zejména genetického základu obou prvků chování. Z toho důvodu jsme v naší laboratoři připravili nové inbrední kmeny, odvozené od divokých populací obou poddruhů. Tyto kmeny s dostatečnou genetickou i fenotypovou proměnlivostí ve sledovaných znacích mohou představovat unikátní nástroj pro studium speciace. V kombinaci s možností testovat získané poznatky přímo v přírodních podmínkách na hybridní zóně mohou pomoci k objasnění genetického základu složitého komplexu chování a jeho významu při vzniku druhů.

Klíčová slova: speciace, etologická izolace, hybridní zóna, slinné Androgen vázící proteiny, močové signály, inbrední kmeny.

## **SPECIATION THROUGH BEHAVIOURAL ISOLATION**

Speciation, the fundamental evolutionary and biological process responsible for the generation of new species and driving force in maintaining biodiversity, has always been a key part of biology. Attempts to understand and explain speciation, this Darwin's "mystery of mysteries", date back to his seminal work published in 1859. Given the remarkable changes in technology and science *per se* during the past 140 years, one would expect that all questions concerning speciation would be resolved. But this is far from being so.

### **Speciation and the genetics of reproductive barriers**

Our present understanding of speciation originates from the Modern Synthesis of Darwinian evolution with Mendelian genetics laid out by Dobzhansky in "*Genetics and the Origin of Species*" (1937), followed by the modern species definition of Ernst Mayr in 1942. Although the debate on species concepts still continues (Hey, 2001), Mayr's biological species concept, where a species is defined as a "group of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups" (Mayr, 1942) remains the most widely held species definition in evolutionary studies. This concept introduces the phenomenon of reproductive barriers, representing a particular set of phenotypic traits preventing species from exchanging genes (Butlin, 2001) that are in most cases measurable end points of a speciation process (Via, 2002). Reproductive barriers can occur either before or after fertilization and different forms of reproductive isolation are not necessarily independent. If we accept the biological species concept the central problem of speciation is to understand the origin and genetics of reproductive barriers that keep species separate (Coyne and Orr, 2004).

Despite the early enthusiasm of founders of the Modern Synthesis we still know very little about the origins and genetics of speciation (Orr et al., 2004). Until now, studies of speciation have been dominated by studies of the geographic context in which this process may occur and by descriptions of different kinds of reproductive isolation. The former demonstrated that reproductive isolation appears to evolve rapidly and usually in allopatry even though the sympatric or parapatric speciation may have occurred as well (reviewed in Via, 2001; Coyne and Orr, 2004). The majority of the latter has mostly involved studies of the genetics of postzygotic isolation largely inspired by Haldane's rule (1922) and the Dobzhansky-Muller model (Dobzhansky, 1937; Muller, 1942). To date, several genes of hybrid male sterility (e. g.

*Odysseus site homeobox (OdsH)* (Ting et al., 1998; Sun et al., 2004)) and hybrid male inviability (e.g. *Hybrid male rescue* gene (*Hmr*) or *Nup96*) potentially causing speciation have been identified, most of them in *Drosophila* species (for review see Orr et al., 2004; Orr, 2005, Noor and Feder, 2006).

The absence of interspecific mating between sympatric taxa and striking sexual dimorphism in sexually reproducing animals led biologists to consider obstacles to fertilisation as the most common cause of reproductive isolation in these organisms (Panhuis et al., 2001; Turelli et al., 2001; Ritchie 2007). Prezygotic barriers appear to evolve in initial stages of speciation and are thought to be one of the most critical components isolating different species (Coyne, 1992; Coyne and Orr, 2004). Species separated only through postzygotic isolation are scarce or do not exist at all (Kirkpatrick and Ravigné, 2002). Even though the prezygotic but postmating isolation is important (Eady, 2001) and in free-spawning animals several putative speciation genes responsible for gamete recognition have been described (e.g. *bindin* in sea urchins (McCarntney and Lessios, 2004) or *lysin* and *VERL* receptor genes in abalone (Galindo et al., 2003; Swanson and Vacquier, 2002)), it seems likely that the behavioural isolation – premating isolation due to divergence of courtship traits and associated preferences – is one of the most common cause of reproductive isolation in animal species (Ptacek, 2000; Panhuis et al., 2001; Ritchie 2007 but see also Moyle, 2007).

### **Behavioural isolation**

Successful mating in sexual animals requires the stepwise completion of a complex series of interdependent events involving courtship displays of both sexes. Only individuals of the same species are able to perform the signal-response sequence necessary to achieve mating whereas this sequence will not be completed successfully in interspecific pairs (Butlin and Ritchie, 1994; Wells and Henry, 1998). Behavioural isolation thus includes all species differences in courtship behaviour that reduce the attractiveness and hence mating between heterospecific individuals during the reproductive period (Butlin, 2001; Coyne and Orr, 2004).

Until now a clear consensus how behavioural isolation may evolve is still missing. The reason for this lack is probably the difficulty with reconciling two conflicting forces: stabilizing selection acting to decrease variance in courtship displays within a species and disruptive selection acting to increase the divergence of courtship traits between isolated groups (Butlin, 1995; Bridle and Ritchie, 2001). An alteration of a trait is likely to reduce the ability to find a

mate and thus any mechanism that allows the coevolution of a trait and the associated preference, unifying both conflicting selection forces, is required.

### The evolution of behavioural isolation

Three main groups of models of the evolution of behavioural isolation have been described so far. The first group assumes the evolution of reproductive isolation to be a by-product of genetic drift or ecological adaptation in allopatry. Random drift and/or different selective pressures in subdivided areas result in the divergence of ecological traits between the populations and if these incidentally affect mate choice signals or preference, then the behavioural isolation evolves as a by-product of this ecological adaptation or random divergence. The resulting selection on mating traits can be direct (due to pleiotropy) or indirect, acting by gene associations with other loci that are under selection (Turelli et al., 2001; Schluter, 2001; Kirkpatrick and Ravigné, 2002; Coyne and Orr, 2004; Nosil et al., 2007).

The second group of models involves the evolution of preference or signal traits by sexual conflict and sexual selection (Turelli et al., 2001). The sexual selection has the potential to lead to rapid divergence between populations, independently on the environment and with high speciation potential as it operates directly on traits involved in mate recognition (Panhuis et al., 2001). Mating preferences in one sex (usually female mate choice) can strongly influence the evolution of mating signals in the opposite sex, resulting in intersexual selection promoting divergence in mate recognition signals (Ptacek, 2000). When females express mating preferences for different values of a trait expressed by males, strong linkage disequilibrium develops between the preference and the trait loci (Kirkpatrick and Ravigné, 2002), leading to the coevolution of a signal trait and associated preference. Behavioural isolation via sexual selection may evolve by two ways. First, selection directly affects either actual preference or a signal trait; in this case, the alleles immediately enhance fitness independently of the genetic background on which they occur. The targets of selection may be, for example, traits improving attractiveness and/or intrasexual competition, higher signal exploitation via sensory drive (Boughman, 2002) or female preference for the best resources and/or higher parental investments. Second, selection acts indirectly on the preference and directly on a signal trait, when a female preference is genetically correlated with selected male traits, like in the good genes theory of runaway sexual selection (reviewed in Turelli et al. 2001; Kirkpatrick and Ravigné, 2002; Coyne and Orr, 2004).

The third group of models is based on selection against disadvantageous heterospecific mating in the secondary contact of partially isolated populations, leading to evolution of behavioural isolation via the adaptive process of reinforcement (Dobzhansky, 1937; Butlin, 1987; 1995; Howard, 1993; Coyne and Orr, 2004; Servedio, 2004). The theory of speciation by reinforcement has long been considered controversial. There is no doubt that reduction of hybrid fitness does generate a selective pressure favouring assortative mating or pronounced divergence of sexual signals in sympatry and associated responses that generate assortment (i.e. the reproductive character displacement, often considered as evolutionary signature of reinforcement), but the conditions under which it can occur have been considered restrictive (Butlin, 1995). The main debate against the reinforcing selection on premating isolation stems from arguments that very strong selection against hybrids, based on at least partial postzygotic isolation, is required to overcome the melting effect of gene flow and recombination. However, a number of recent theoretical studies have demonstrated plausibility of this process under natural conditions (reviewed in Turelli et al., 2001, and Kirkpatrick and Ravigné, 2002; Marshall et al., 2002). Natural selection acts on genes that cause low hybrid fitness and the assortative preference thus evolves indirectly via linkage disequilibrium. In this case the indirect selection on preference may be relatively effective as in hybridising species the selection against hybridisation and genetic associations may be very strong (Kirkpatrick and Ravigné, 2002). Recent studies show reinforcement may occur with or without gene flow between diverging populations (Servedio, 2000; 2004) and in specific situations selection against hybrids may exist even without intrinsic postzygotic isolation (for example in cases where hybrids are less fit in parental ecological niches) (Servedio, 2004; Nosil et al., 2007). There is a growing evidence of the occurrence of reinforcement in nature (reviewed in Servedio and Noor, 2003; Hoskin et al., 2005, Smadja and Butlin, 2006) but its role in the speciation still remains uncertain.

Many selective processes may contribute to the evolution of behavioural isolation and their relative importance is a major question in current speciation research. In spite of this the disruption of the complex set of behavioural traits leading to recognition and successful mating only within conspecifics has been shown to be an efficient barrier to gene flow between closely related species and some progress has been made also in the identification of genes involved in behavioural isolation.

### Examples and genetics of behavioural isolation

The occurrence of behavioural isolation has been documented either through a vast array of laboratory mate choice experiments revealing existence of assortative mating among conspecifics or in nature where this isolation can be inferred from the divergence of specific sensory systems in closely related species living in sympatry. During the last few decades, evidence has been accumulating of the divergence in visual, acoustic, olfactory or tactile sensory systems in a variety of different taxa ranging from insects to mammals (reviewed in Howard 1993; Wells and Henry 1998; Ptacek, 2000; Coyne and Orr, 2004; Ritchie 2007; Smadja and Butlin 2008; Rocha and Bowen, 2008). While these studies have suggested a primary role of behavioural isolation in speciation, it is much harder to identify which traits are crucial as reproductive barriers and then to study their genetic background (Coyne and Orr, 2004). Indeed, such studies are scarce and, obviously, highly biased toward model organisms, especially *Drosophila*.

In fruit flies the courtship and mating behaviour may involve visual, acoustic and olfactory systems (Markow and O'Grady, 2005) acting either separately or together to strengthen and complete the transmitted information (Greenspen and Ferveur, 2000). Until now, two groups of putative genes driving speciation through behavioural isolation have been described. The *desaturase2* gene is responsible for different profiles of cuticular hydrocarbons in different populations of *D. melanogaster*, which serve as olfactory contact signals leading to discrimination and assortative mating between the populations (Ferveur, 2005). Similarly, one locus responsible for female assortative preference of male courtship wingbeat has been described in *D. pallidosa* and *D. annanasae* (Doi et al., 2001) and the *period* gene affecting different courtship songs was identified in *D. melanogaster* and *D. simulans* (Ritchie et al., 1998).

Even though the genetic studies of behavioural isolation are limited and biased to one genus, they allow drawing several conclusions. The genetic basis of behavioural isolation may be relatively simple, involving changes in a few genes, usually distinct between both sexes. The signal divergence is usually asymmetric and seems to evolve rapidly either through major shifts or by more gradual changes (reviewed in Coyne and Orr, 2004, Ritch, 2007 and Smadja and Butlin, 2008). However, detailed studies involving diverse species from different taxa are required to draw clearer picture of genetic architecture of behavioural isolation.

During the last decade progress in genomic techniques has opened a possibility to study the genetics of behavioural isolation also in other model groups: several quantitative trait loci affecting morphological or colour differences in African cichlids (Haesler and Seahausen,

2005) or courtship songs differences in Hawaiian crickets have been identified (Shaw and Parsons, 2002) as well as several candidate loci coding different female pheromones involved in behavioural isolation of different moths species or corn borers (*Ostrinia*) (reviewed in Ritchie, 2007, and Smadja and Butlin, 2008). The sensory genes are now being characterised in a range of species as a variety of genome sequences has become available and the genomics of sensory systems can now help to unravel the molecular mechanisms of species communication. The accessibility to sets of gene families involved in signal production, reception and perception may present a new approach extremely powerful in search for putative speciation genes and in combination with classical genetic analyses may provide new opportunities to detailed dissection of genetics of this species specific modality up to insights to the genetic architecture of complex behaviour and its role in speciation (Noor and Feder 2006, Smadja and Butlin 2008).

### **Traditional and modern approaches to study isolation barriers**

Evolutionists have performed an impressive number of studies on biogeography, ecology and genetics of speciation unravelling a good base about the evolution of reproductive barriers (Coyne and Orr, 2004). Despite this progress, only very limited number of potential speciation genes have been identified and these are nearly exclusively limited to insects or sea-marine invertebrates. The reason is simply in the contrast of attempts of naturalists to study a continuous process of speciation using as a tool different species, i.e. taxa that are by definition reproductively isolated units without a continuous gene flow (Orr, 2005), where the speciation process has been completed. As a consequence, a large set of fundamental questions about the genetics of speciation has been neither solved nor even addressed.

A traditional approach to study the genetics of speciation has used either laboratory or garden hybridisation studies or comparative studies of closely related taxa. The former attempted to identify the number, type and distribution of genes contributing to phenotypes preventing gene flow between the taxa, whereas the latter examined patterns of differentiated genomes within and between closely related or diverging taxa (reviewed in Coyne and Orr, 2004; Noor and Feder, 2006). However, the implications of these studies are limited by unclear significance of genes or traits identified in the laboratory as causing reproductive isolation also for natural conditions involving gene flow, interactions with extrinsic ecological conditions, and a joint action of different selective forces. Survey of natural hybrid

populations is therefore needed to test the significance and strength of different isolation barriers and to make inferences about the genetics of reproductive isolation in nature.

Hybrid zones, i.e. regions where distinct populations meet, mate and produce hybrid progeny, have sometimes been called “natural laboratories” (Hewitt, 1988) or “windows to evolutionary processes” (Harrison, 1990). They can be seen as genomic core facilities providing hybrid material recombined for many more generations that are obtainable in the laboratory and tested directly under natural conditions considering all aspects of hybrid fitness (Barton and Hewitt, 1985). Recent advances in molecular and genomic techniques allowed extending of speciation studies to non-model genetic organisms and until now a long list of hybrid zone studies addressing speciation genetic questions have been performed in a variety of distinct taxa (for review see Barton and Hewitt, 1989; Harrison, 1990; Jiggins and Mallet, 2000). They yielded estimates of the number of genes that contribute to barriers to gene flow and insights to the forces driving their evolution in respect to balance between selection and migration and the relative importance of genomic incompatibilities, population demography and ecology in maintenance of genetic integrity of different taxa (Barton and Hewitt, 1985; 1989; Barton and Gale, 1993; Rieseberg et al., 1999; Jiggins and Mallet, 2000; Raufaste et al., 2005; Macholán et al., 2007).

The development of ever-more sophisticated analytical methods (allowing rigorous testing of statistical models and phylogenetic reconstructions) together with advanced molecular techniques (including whole-genome databases of model organisms, expression analysis and direct gene manipulations) have rendered a new impetus to refining our understanding the genetic architecture and permeability of species boundaries (Noor and Feder, 2006). They lead to description of candidate regions of higher differentiation – “islands of speciation”, whose contribution to phenotypes involved in reproductive barriers may be tested either in the laboratory or under field conditions. This candidate-gene approach may be extremely powerful in combination with classical experimental approaches in description of new putative speciation genes especially when the target animal is the laboratory model organism with described genome sequence and at the same time it is hybridising in nature. Until now, only two organisms fulfil these strict conditions; the fruit flies of the genus *Drosophila* and the house mouse.



## THE HOUSE MOUSE: MODEL FOR GENETIC AND EVOLUTIONARY STUDIES

### From nature to lab and back again

The house mouse (*Mus musculus*) has been living in intimate association with humans since the dawn of their civilization. It is thought that this commensal habit first appeared in the Fertile Crescent during the Epipaleolithic (Boursot et al., 1993; Silver 1995) when the first stable human settlements have opened a new ecological niche for this small mammal, rendering it a possibility to escape from competition with other species (Auffray et al., 1988). Obviously, such characteristics as opportunism in habitat and food preferences as well as the high reproductive rate and generation turn-over have made the house mouse pre-adapted to the synanthropic association with humans and to become the second most successful mammal species – after the man – currently living on Earth (Berry and Scriven, 2005). In addition, the high reproductive rate and ease with which it can be kept, handled and bred in captivity seem to predetermine this animal to be an extremely useful laboratory model organism and indeed, over the past century the mouse has become a premier mammal model system for a vast array of biomedical, ecological, and evolutionary surveys. The knowledge of the whole sequence of its genome (Mouse Genome Sequencing Consortium, 2002) and availability of dense genetic maps have brought about another impetus to these research activities.

From the perspective of genetics, the mouse has become an important model organism immediately after the rediscovery of Mendel's laws in 1900. Indeed, several authors have even suggested that Mendel himself first arrived at his principles of heredity by virtue of his experiments on mice yet was forced by the church authorities to get rid of these “voluptuous and libidinous animals having sex” (Sturtevant, 1965; Silver, 1995; Paigen, 2003; Berry and Scriven, 2005). Notwithstanding this hypothesis to be true or not, as early as in 1902 Lucien Cuénot proved, after two years of work on mice, that Mendelism applied to animals as well as plants (Berry and Scriven, 2005).

From the very beginning, the new discipline (and mouse genetics in particular) was intimately connected with development of inbred strains of laboratory mice (a strain is defined as inbred after at least 20 generations of strict brother-sister mating). This work has been pioneered by C. C. Little who established a first inbred strain in 1909 (later called DBA after three characteristic coat colour mutations: *dilute*, *brown*, *non-agouti*). After the First World War he moved to the Carnegie Institution Department of Genetics at Cold Spring Harbor (today Cold Spring Harbor Laboratory) where he continued, together with L. C.

Strong, in mouse breeding experiments and development of new inbred strains. In 1929 Little established the Roscoe B. Jackson Memorial Laboratory (now The Jackson Laboratory) in Bar Harbor, Maine, which played the crucial role in the development of the mouse into the leading model for biomedical research and which has grown into the largest centre of mouse genetic research offering hundreds of strains as animal models (<http://jaxmice.jax.org/>).

Although the laboratory mouse provides a powerful tool for understanding many biological processes (Davisson and Linder, 2004), an animal strictly inbred for many generations and reared under controlled conditions loses its natural variability in genotype (reduced to one allele-state) but also in phenotype such as behavioural and physiological reactions (Berry and Scriven, 2005). Moreover, the “classical” mouse strains harbour a limited variety of natural genetic polymorphisms as they are derived from a small pool of ancestors (Guénet and Bonhomme, 2003). On the other hand, these strains have been shown to represent a mixture of genomes from different mouse taxa (Bishop et al., 1985; Bonhomme et al., 1987; Wade et al., 2002; Frazer et al., 2004). Laboratory mice thus can render only limited information about the real world, thus potentially hampering the inferences about the evolutionary processes in nature. This relative lack of genotype and phenotype variation can be overcome either by developing an array of new inbred strains derived directly from a wide variety of mouse species and/or subspecies or by focusing on wild mouse populations as an invaluable source of polymorphism (Guénet and Bonhomme, 2003; Piálek et al., 2008 – Supplement 3). In the last decade several different inbred strains have been derived from wild populations of different mouse taxa and their phenotypes and genetic polymorphisms are going to be described (Gregorová and Forejt, 2000; Campino et al., 2002; Fernandes et al., 2004; Jansa et al., 2005; Bogue et al., 2007; Piálek et al., 2008 – Supplement 3). These wild-derived inbred strains present a new direction in mouse genetic research and in combination with studies on natural populations they can be seen as a unique tool to get insight in genetics of speciation process occurring in nature.

### **Natural hybridization in house mice – an exceptional tool for evolutionary biologists**

House mice of the *Mus musculus* complex represent at least four morphologically very similar, but genetically distinct forms referred to in literature either as distinct species or subspecies (Fig. 1): *M. (musculus) musculus*, occurring from central Europe to the Far East; *M. (m.) domesticus*, with the native range including western and southern Europe, North Africa and the Middle East and spreading with humans to the Americas, Australia and to

Africa south of Sahara; *M. (m.) castaneus* occupying south-eastern Asia; and *M. (m.) gentilulus* from Yemen (Boursot et al. 1993; Prager et al. 1998; Guénet and Bonhomme 2003). Recently, a new lineage has been discovered on the Yemeni island of Socotra (M. Macholán, pers. com).



Fig.1: The geographic distribution of the house mouse subspecies of the *Mus musculus* complex. For details about different subspecies, especially the hybrid form “*molossinus*” see the text. A new lineage discovered on the Yemeni island of Socotra (M. Macholán, pers. com) is not presented. White area with the question marks represents a complex situation in south-central Asia where mice were found to possess predominantly *castaneus*-type of mtDNA, either *musculus*-type or *domesticus*-type of Y chromosome and an extensive variation of autosomal genes. Mice of the Americas, Australia and Africa south of Sahara have been imported by humans during the colonization. The dotted white line indicates *domesticus/musculus* hybrid zone in Europe, which is a centre of intensive speciation studies (modified from Guénet and Bonhomme, 2003).

These taxa present an excellent system for evolutionary studies as they show various levels of reproductive isolation, and can interbreed in the lab and partially also in nature, forming areas with viable hybrids and backcrosses for several generations. A large-scale intergradation between *musculus* and *castaneus* in the Far East and Japan gave rise to a hybrid form originally described as *M. m. molossinus* (Yonekawa et al., 1988). Another example is represented by hybrid zones, long and narrow areas with individuals of hybrid and

recombined origin spread along the contact border between two distinct forms (e.g. *domesticus/musculus* hybrid zone in Europe or *musculus/castaneus* hybrid zone in Central China). By far the best documented and most thoroughly studied is the *musculus/domesticus* hybrid zone forming about 2500 km long and 20–30 km wide contact front running across the Jutland peninsula and from East Holstein through central Europe and the Balkans to the Black Sea coast (Boursot et al., 1993; Sage et al., 1993; Macholán et al., 2003). This zone has been in the centre of interest of evolutionary biologists over last 50 years.

Despite the long-term and intensive studies of this hybrid zone we still know very little about the causes and mechanisms that keep both subspecies separate. Until now, only several lines of indirect evidence suggest that selection is acting against hybrids, nonetheless a direct proof is still missing. For example, hybrid male sterility and partial female sterility have been described in different crosses of laboratory or wild populations (Forejt and Ivanyi, 1974; Forejt, 1996; Oka et al., 2004; Storchová et al., 2004; Britton-Davidian et al., 2005; Vyskočilová et al., 2005; Good et al., 2008; Vyskočilová and Piálek, in prep.). In addition, higher parasite susceptibility has been found in individuals from the centre of the hybrid zone (Sage et al., 1986; Moulia et al., 1991) whereas limited introgression of sex chromosome markers as compared to autosomes has been shown across five studied transects (Vanlerberghe et al., 1986; Tucker et al., 1992; Dod et al., 1993; Dod et al., 2005; Raufaste et al., 2005; Macholán et al., 2007; Macholán et al., in press – Supplement 5). Finally, recent genome-wide mapping studies have highlighted several potential candidates for “speciation genes” some of which being associated with olfaction, pheromone response or other behavioural aspects of reproduction (Haar, 2006; Teeter et al., 2008), suggesting potential importance of behavioural isolation between both mouse subspecies as previously proposed by Karn et al. (2002), Smadja and Ganem (2002) or Dod et al. (2005).

### **Behavioural isolation in the mouse hybrid zone**

Because of predominantly nocturnal life, communication among house mice is mediated mainly through olfaction. Olfactory cues, usually specific chemosignals or by-products of metabolic processes, convey complex information including sex, reproductive and health status, ownership or competitive ability, but also individual identity such as genotype, familiarity, kinship or genetic relatedness (review in Beauchamps and Yamazaki, 2003; Brennan and Kendrick, 2006). In the context of the *musculus-domesticus* recognition, the ability to discriminate and choose conspecifics based on odorant stimuli has been

repeatedly demonstrated (Laukaitis et al., 1997; Christophe and Baudoin, 1998; Munclinger and Frynta, 2000; Talley et al., 2001; Smadja and Ganem, 2002; 2005; Smadja et al., 2004; Bímová et al., 2005 – Supplement 2; Ganem et al., 2008). Interestingly, these studies have revealed significant differences in discrimination and choosiness both between the two subspecies and sexes.

According to theory of sexual conflict and higher reproductive cost for females (Panhuis et al., 2001), we would expect to find stronger assortative preferences in females of both subspecies, as the cost of heterosubspecific mating should be the same for females on both sides of the hybrid zone. However, recent studies have shown assortative preferences to be stronger in males than in females (Piálek et al., 2008 – Supplement 3; Ganem et al., 2008; Bímová et al., in press – Supplement 4); moreover, both males and females of *M. (m.) musculus* are more choosy than males and females of *M. (m.) domesticus* (Christophe and Baudoin, 1998; Smadja and Ganem, 2002; 2005; Smadja et al., 2004; Bímová et al. 2005 – Supplement 2; Piálek et al., 2008 – Supplement 3; Ganem et al., 2008; Bímová et al., in press – Supplement 4).

What is the reason for these unexpected differences? One of possible explanations could be the difference in male aggressiveness between both subspecies. Although the level of male aggression may differ across populations of a single subspecies (Frynta et al., 2005) all *domesticus* males are more aggressive than *musculus* males so that when tested against each other, the former always win the encounter and dominate the latter (Thuesen, 1977; van Zegeren and van Oortmerssen, 1981; Munclinger and Frynta 2000; Frynta et al., 2005; Piálek et al. 2008 – Supplement 3). It seems that the *musculus* males themselves elicit fights only as owners of the territory, probably just to its defence (Piálek et al., 2008 – Supplement 3). House mice usually live in small local populations, demes, with a hierarchical structure in both males and females being established on the basis of aggressive encounters (Anderson and Hill, 1964; Bronson 1979; Berry and Scriven, 2005). Genetic studies have revealed that more than 70% of pups in a deme are offspring of a dominant male and that females mate almost exclusively with that dominant male in the most fertile period of the oestrus cycle (Bronson 1979; Drickamer et al., 2000; Dean et al., 2006). Higher male aggressiveness can thus lead to a dominant position in a deme, higher access to reproduction and hence higher fitness (DeFries et al., 1970). In such a situation, one can imagine that when coming in contact, more aggressive and non-choosy *domesticus* males would disperse into the *musculus* territory replacing *musculus* males. This process should result in higher introgression of *domesticus* alleles across the zone and the movement of the whole contact front into the

*musculus* territory. However, evidence for any movement of the hybrid zone is still lacking and only limited introgression of alleles in *domesticus-musculus* direction have been reported from Denmark (Dod et al., 1993; Raufaste et al., 2005) and Saxony (Tucker et al., 1992; Božíková et al., 2005; Teeter et al., 2008). Conversely, in the Czech-Bavarian transect the introgression of mtDNA (Božíková et al., 2005) and Y chromosome markers (Munclinger et al., 2002; Macholán et al., in press – Supplement 5) were found to be in the opposite direction and it seems that the reason for this pattern is probably the genomic conflict rather than behavioural strategies of both subspecies (Macholán et al., in press – Supplement 5; Bímová, unpublished data). Theoretically, stronger assortative mating may have evolved in *musculus* males as a counterstrategy against the invasion of more aggressive *domesticus* males but again, there is no evidence supporting this hypothesis. Nevertheless, behavioural isolation in the *musculus/domesticus* hybrid zone seems to be a complex phenomenon involving both male-male competition and assortative mating, though the extent to which it contributes to the process of speciation in the house mouse complex remains undetermined.

### **In-depth description of behavioural isolation components**

To analyse the role of both behavioural strategies (aggressiveness and assortative mating) on a dynamics of the house mouse hybrid zone we should first describe both phenotypes and their genetic determination and consecutively analyse these traits and their interactions in natural populations in the area of secondary contact between the two subspecies. The experiments using cross-fostering design confirmed the genetic inheritance and only negligible role of postnatal maternal environment on both observed behavioural traits (Ďureje and Bímová, unpublished data). During the last decade a progress has been made in the description of genetic basis of male aggressiveness and several quantitative trait loci (QTLs) affecting this trait has been identified on different chromosomes (Brodkin et al., 2002; Roubertoux and Le Roy-Duflos, 2001; Roubertoux et al., 2005). Interestingly, these studies corroborate results of previous studies indicating that the Y chromosome most probably plays less important role in determination of male aggression that was previously thought (Volfová et al., 2002; Bímová, unpublished data). On the other hand, mapping and identification of QTLs for assortative mating is more complex since we first need to describe both parts of the recognition system, i.e. the signal and the preference for that signal.

Until now studies on assortative mate choice in house mice (see above) have been focused mainly on ability to discriminate and prefer mates of the same subspecies rather than to

determine genes and signal components responsible for the preference. A notable exception are studies of an amazing system of salivary androgen binding proteins (ABP) carried out in the laboratory of R. C. Karn at the Butler University in Indianapolis, Indiana (now at the University of Arizona, Tuscon). The results of these studies led Karn and his collaborators to propose the ABP system to be responsible for prezygotic isolation between various house mouse subspecies (Laukaitis et al., 1997; Talley et al., 2001; Karn et al., 2002). However, recent studies from the *musculus/domesticus* hybrid zone, both behavioural (Bímová et al., 2005 – Supplement 2) and genetic (Dod et al., 2005; Macholán et al., in press – Supplement 5), does not seem to be in agreement with the predictions. The genetic studies indicated that the transition of *Abpa* subspecies specific alleles across the hybrid zone is more similar to the transition of neutral markers than to selected ones and thus it does not seem that the selection is acting against *Abpa* introgression as would be expected for a trait involved in premating isolation (Dod et al., 2005; Macholán et al., in press – Supplement 5). Accordingly, behavioural analyses did not revealed significant and consistent ABP-based homosubspecific preferences in both allopatric and parapatric populations even though the whole pattern of preferences were skewed toward homosubspecific ones mostly in *M. (m.) musculus* populations. Moreover, the time spent by sniffing the homosubspecific ABP-signals was longest in populations from the edges of the hybrid zone that may potentially indicate the pattern of reinforcement acting on assortative mating in this part of the hybrid zone (Bímová et al., 2005 – Supplement 2). Thus we can not reject the role of ABP, but it seems to be only one element of a more complex signalling system, most probably acting in transmitting the information between the animals in close contact (Bímová et al., in press – Supplement 4). In addition, when compared to other potential signalling components saliva and especially salivary ABP elicited weaker preferences than stimuli that have the capacity to carry a signal for extended periods under natural conditions such as urine and faeces (Bímová et al., in press – Supplement 4; Bímová, unpublished data). This finding corroborates results of studies by C. Smadja and G. Ganem (Smadja and Ganem, 2002, 2005; Smadja et al., 2004, Ganem et al., 2008) showing that subspecies-specific recognition occurs through urinary signals and that this recognition have been reinforced after the secondary contact of the two subspecies (Smadja and Ganem, 2005; 2008; Bímová, unpublished data). Thus it seems that urinary pheromones, either volatiles coded for by MHC genes or carried and released by major urinary proteins (MUPs), are the most important substances in mouse signalling (Novotny et al., 2003; Brennan and Kendrick, 2006).

Contrary to genetic similarity primarily manifested by MHC and background genes (Penn, 2002; Willse et al. 2006, Röck et al., 2007), polygenic and highly polymorphic MUPs may present a more reliable identity signature (Hurst et al., 2005; Cheetham et al., 2007; Sherborne et al., 2007; Thom et al., 2008) reflecting both genetic and temporal status information (Stopka et al., 2007; Rusu et al., 2008). MUPs thus seem to be the most likely candidates for subspecies-specific indicators (Beynon et al., 2007, Stopková et al., 2007). However, direct confirmation of such a role of MUPs is still missing though recently species-specific (Robertson et al., 2007) and subspecies-specific (Stopková et al., 2007) differences in expression of MUPs and their concentration in urine have been described and analyses of subspecies-specific MUP profiles in the house mouse hybrid zone is in progress (Janotová, unpublished data; Ganem, unpublished data).

Finally, when analysing the genetics of different components of assortative mating, we should look also on the receptor part of the recognition system and to study possible differences between mouse subspecies, which seems to be marginalised or avoided in most studies. In mammals pheromones are perceived by two olfactory systems, the main olfactory system (MOV) and the vomeronasal systems (VNS), both more or less used to process the odorant information from the environment and conspecifics (Brennan and Keverne 2004; Grus and Zhang 2008). Over the past several years more than a thousand of genes coding for odorant receptors of both systems has been described in the mouse and some progress has been made in the identification of their structure, expression and function (Rodriguez et al., 2002; Grus and Zhang 2004; 2008). Using the advantage of growing genomic databases of a set of genes involved in mouse olfactory communication, together with availability of wild-derived inbred strains and advanced molecular and statistical techniques for analyses of natural populations, open the door to studies on the molecular mechanisms and origins of subspecies-specific pheromone communication. By linking genetics, chemistry and physiology on the one side with ecology and evolution on the other, the mouse presents a unique model providing the opportunity to unravel the genetic bases of such complex behaviour and its role in reproductive isolation and speciation.



## CONCLUSIONS

In my thesis I studied the role of behavioural isolation as an efficient premating barrier contributing to or completing the speciation between two house mouse subspecies, *M. m. musculus* and *M. m. domesticus*. I found that there is a strong divergence in both the signal and preference parts of the subspecies-specific recognition system between the two subspecies. From the tested signal systems, salivary Androgen binding proteins do not seem to act as an efficient barrier to noticeably impede the gene flow across the house mouse hybrid zone, but they may participate in subspecies-specific recognition as transmitters of information between interacting animals in close contact. Conversely, urinary signals elicit significant assortative mating preferences and most probably may be the most important subspecies-specific indicators. Especially recent growing evidence of the importance of major urinary proteins (MUPs) supports the hypotheses that MUPs may present the hot candidates for potential isolation traits. Interestingly, our results indicate that males may participate more in behavioural isolation than females, contrary to a generally accepted view, males were the more choosy sex in all performed experiments.

Based on our results I can conclude that behavioural isolation in the mouse hybrid zone may play an important role as a barrier to gene flow; this barrier is asymmetric between both subspecies, involves both males and females and should be seen as a complex involving at least two different strategies: the assortative mating on the one side and male aggressiveness on the other. However, the extent to which both strategies participate in the mouse speciation remains uncertain and needs further analyses mainly of the genetic architecture of involved behavioural phenotypes. For that purpose we prepared eight new inbred strains, derived from wild populations sampled on both sides of the hybrid zone (one on the *domesticus* side and two on the *musculus* side) representing genomes of both subspecies. The genetic and phenotype analyses confirmed sufficient genomic variation between these strains and subspecies-specific differences in phenotype traits potentially involved in reproductive isolation (e.g. morphology, reproductive performance, and male sterility in crosses of two of our strains with “classical” inbred strains, assortative mate choice preferences, male aggression and *in vitro* immune response). Furthermore, on average 82% of 361 microsatellite markers scored within these strains were diagnostic for either the *musculus* or *domesticus* strains representing a dense genetic map of diagnostic markers regularly distributed across whole genome. These strains may present a unique tool for analyses of quantitative trait loci

(QTL) affecting phenotypes involved in reproductive barriers and help to unravel the genetic basis of speciation.

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