Development of Unique House Mouse Resources Suitable for Evolutionary Studies of Speciation

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Abstract

Two house mouse subspecies, *Mus musculus domesticus* and *Mus musculus musculus*, form a hybrid zone in Europe and represent a suitable model for inferring the genes contributing to isolation barriers between parental taxa. Despite long-term intensive studies of this hybrid zone, we still know relatively little about the causes and mechanisms maintaining the 2 taxa as separate subspecies; therefore, to gain insight into this process, we developed 8 wild-derived inbred house mouse strains. In order to produce strains as pure *domesticus* or *musculus* genomes as possible, the individuals used to establish the breeding colony for the 3 *domesticus* and 2 of the *musculus* strains were captured in the Czech Republic from wild populations at extreme western and eastern edges of the subspecific contact zone, respectively. The remaining 3 *musculus* strains were bred from mice captured about 250 km east of the hybrid zone. Genetic analysis based on 361 microsatellite loci showed that 82% of these markers are diagnostic for either the *musculus* or the *domesticus* strains. In order to demonstrate the potential utility of this genetic differentiation in such strains, phenotypic variation was scored for 2 strains from opposite edges of the hybrid zone and significant differences in morphology, reproductive performance, in vitro immune responses, mate choice based on urinary signals, and aggressiveness were found. In addition, the 3 strains derived from *musculus* populations far from the hybrid zone display significant differences in polymorphism in hybrid male sterility when crossed with the laboratory strains C57BL/6 or C57BL/10, which have a predominantly *domesticus* genome. Although further studies will be necessary to demonstrate intersubspecific differences, all analyses presented here indicate that these newly developed house mouse strains represent a powerful tool for elucidating the genetic basis of isolation barriers in hybrid zones and for studying speciation in general.

One of the central tenets of evolutionary biology lies in the description and understanding of the barriers preventing gene flow between 2 incipient species. A model organism highly suitable for this purpose is the house mouse, *Mus musculus*. Its 2 subspecies, *Mus musculus domesticus* and *Mus musculus musculus* interbreed under natural conditions and in Europe form a narrow hybrid zone about 2500 km long stretching from the Jutland Peninsula in Denmark to the Black Sea in Bulgaria (Macholán et al. 2003). Two basic strategies can be used to study speciation in these wild populations. The first approach is to study free-living mice in those geographic regions where these 2 subspecies meet and hybridize: by estimating the strength of selection acting on a set of markers differing between the parental populations, it is possible to identify some of the genomic regions that contribute to the creation of barriers to gene

flow and, hence, may harbor the genes responsible for speciation (Payseur et al. 2004; Dod et al. 2005; Payseur and Nachman 2005; Raufaste et al. 2005; but see Macholán et al. 2007). Unfortunately, high levels of genetic polymorphism in natural populations means that this approach is extremely costly and time consuming because it requires the identification of a large number of diagnostic markers, that is, those with different alleles fixed in each parental taxa. Alternatively, we propose that inbred strains specifically selected for certain traits useful for studying speciation and with reduced overall genetic variation could be used to perform between-strain crosses and backcrosses, so that genetic incompatibilities that potentially hamper gene flow and preventing intermixing of the 2 parental genomes could be detected. Comparing the results from these 2 approaches could prove decisive in the understanding of the evolutionary processes driving speciation.

Nearly 450 laboratory strains of the house mouse and their variants have already been characterized and listed (Festing 1996). However, although these variants have proved to be of critical importance for the understanding of many biological processes (Davisson and Linder 2004), existing laboratory mouse strains are not particularly useful for mapping speciation genes. First, most "classical" mouse strains represent a mixture of various mouse genomes (Wade et al. 2002; Frazer et al. 2004; Wade and Daly 2005), and the assignment of different parts of a particular mouse strain genome to either the *musculus* or the *domesticus* subspecies cannot be ascertained without identification of the ancestral polymorphisms present before lineage splitting and the mutations induced during long-term inbreeding. Second, despite the high number of variants, the majority is descended from a limited number of mouse strains and represents mutant types of a spontaneous or induced origin. Instead, the selection of house mouse strains from wild individuals reduces natural genetic variation to a 1-allele state at many loci for each strain and this interstrain genetic variation can be easily detected and assigned to either the *M. m. domesticus* or the *M. m. musculus* genome. Finally, mouse stocks inbred for at least ~20 generations also display more uniform phenotypic traits. This fact facilitates the definition of phenotypic classes and the detection of associations between genotypes and phenotypes using quantitative trait loci (QTLs) mapping.

In the present paper, we describe the development of 8 wild-derived house mouse strains with genetic and phenotypic differences for inferring the evolutionary processes occurring in situ in this hybrid zone and to elucidate the genetic basis of hybrid sterility and speciation genes in general. We conclude that both genetic and phenotype traits differ among strains and genomes and are potentially suitable for genetic dissection in QTL studies.

Materials and Methods

Source Populations of House Mouse Strains

The founders of all inbred strains were captured from wild populations of the house mouse in the Czech Republic and
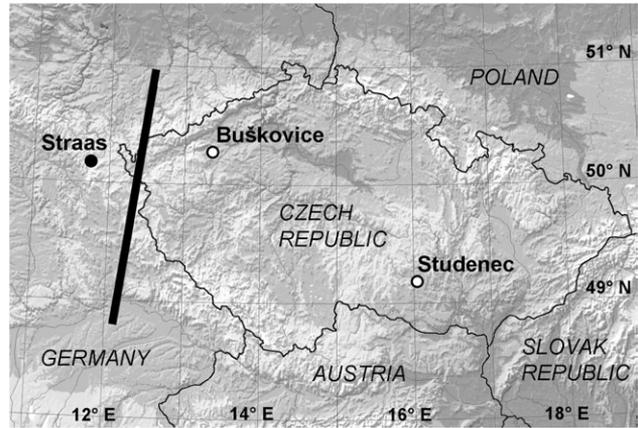
Figure 1. Localities where wild mice were collected (founders of inbred strains described in this paper): open circles, *musculus* populations; closed circle, *domesticus* population; and black line, position of the center of the hybrid zone between *Mus musculus domesticus* and *Mus musculus musculus* in this area. The map was created using Microsoft Encarta Premium Suite (2003).

Germany (Figure 1). Five of these strains were developed from individuals considered to have pure *M. m. musculus* genomes and 3 from individuals with a *M. m. domesticus* genome. Three inbred lines selected for alternative phenotypes regarding sterility, when mated with the laboratory mice of C57BL/6 or C57BL/10 (see details in Results), were derived from wild *M. m. musculus* caught in November 2000 in the village of Studenec, Moravia, Czech Republic (Figure 1 and Table 1), which is 250 km east of the *musculus–domesticus* hybrid zone (populations in this area are considered to be pure *musculus* mice [Munclinger et al. 2002; Božíková et al. 2005]). The founders of the 3 *domesticus* and 2 other *musculus* strains were captured at the extreme western and eastern edges of the contact zone between the mouse subspecies about 50 km on either side of the hybrid zone center in villages of Straas and Buškovice, respectively (Figure 1; Munclinger et al. 2002; Macholán et al. 2007). Previous statistical analyses have indicated that the width of the multilocus autosomal and the compound X chromosome cline is about 9.6 km and 4.6 km, respectively, giving the estimate of the strength of the central barrier as about 20 km (Macholán et al. 2007). This estimate suggests that very few alleles will be able to cross and penetrate the contact zone for more than a few kilometers. Some introgression of the *musculus* and *domesticus* genomes might be expected in these 2 source villages due to their position on either side of the hybrid zone; however, because they are 50 km from the center of the zone, we expect that the proportion of introgressed alleles will be very low and these populations can be considered "pure." However, detailed genetic analyses of the 3 source populations are presented here in order to confirm this assumption (see Genetic Analyses below).

Table 1. Origin of wild-derived strains maintained in the Department of Population Biology, Studenec, Czech Republic

Subspecies	Strain	Dam	Sire	Date of capture	Locality[a]	Country[b]	Latitude (N)	Longitude (E)	Generation as of 31 December 2006[c]
musculus	STUF	JPC 2802	JPC 2804	5 November 2000	Studenec	CZ	49°12′	16°04′	F_{23}
musculus	STUP	JPC 2814	JPC 2824	5 November 2000	Studenec	CZ	49°12′	16°04′	F_{26}
musculus	STUS	JPC 2821	JPC 2822	6 November 2000	Studenec	CZ	49°12′	16°04′	F_{17}
musculus	BUSNA	JPC 2847	JPC 2851	21 November 2000	Buškovice	CZ	50°14′	13°22′	F_{23}
musculus	BULS	JPC 2856	JPC 2852	21 November 2000	Buškovice	CZ	50°13′	13°23′	F_{19}
domesticus	STRA	JPC 2705	JPC 2711	27 September 2000	Straas	D	50°11′	11°46′	F_{20}
domesticus		JPC 2706	JPC 2713	27 September 2000	Straas	D	50°11′	11°46′	—
domesticus	STRB	JPC 2790 F_1	JPC 2721	12 January 2001	Straas	D	50°11′	11°46′	F_{17}
domesticus	STLT	JPC 2788 F_1	JPC 2716	12 January 2001	Straas	D	50°11′	11°46′	F_{19}

[a] see Figure 1

[b] CZ = Czech Republic; D = Germany

[c] F_x indicates the xth generation of offspring born in captivity.

Housing and Breeding of Animals

The inbred strains are presently maintained at the Department of Population Biology, Studenec, Czech Republic. Ten wild parental pairs per sample population from Buškovice (*musculus*) and Studenec (*musculus*) and 3 parental pairs from Straas (*domesticus*) were used to set up the breeding colony founded at this facility. Based on reproductive performance, the *musculus* samples were reduced to 3 lines per population at the 10th generation born in captivity (F_{10}). In general, each generation was derived from 1 of 3 pairs of mice kept per line. The young were weaned at 20 days of age and bred in brother–sister pairs. All individuals were kept in Perspex cages at 20–22 °C on a 14/10-h light/dark cycle. Pelleted food (ST1, VELAZ, Prague, Czech Republic) and water were available *ad libitum*.

The breeding facility in Studenec has been licensed for keeping small mammals according to Czech law since 2000. Mice are kept in an open breeding system without barriers. All animal experiments followed protocols approved by Institutional and National Committees for Animal Welfare.

Genetic Analyses

Genomic DNA was extracted from individuals using DNeasy Tissue Kit (Qiagen, Hilden, Germany) from ethanol-preserved spleen tissue. Wild mice were assigned either to the *domesticus* or to the *musculus* subspecies using 1 extranuclear, 3 sex-linked, and 7 autosomal markers known a priori to be diagnostic for both house mouse subspecies: 1) the presence (*domesticus*)/absence (*musculus*) of the *Bam*HI restriction site in the reduced form of nicotinamide adenine dinucleotide dehydrogenase-1 (*mtNd1*) gene of the mitochondrial DNA (Boursot et al. 1996; Munclinger et al. 2002; Božíková et al. 2005); 2) the presence (*musculus*)/absence (*domesticus*) of a deletion located within the zinc finger protein 2 (*Zfy2*) gene on the Y chromosome (Nagamine et al. 1992; Orth et al. 1996); 3) and 4) the presence (*domesticus*)/absence (*musculus*) of the B1 insertions in the Bruton agammaglobulinemia tyrosine kinase (*Btk*) and testis-specific (*Tsx*) genes

on the X chromosome (Munclinger et al. 2002, 2003); 5) the presence/absence of polymerase chain reaction (PCR) products using subspecies-specific primers within the androgen-binding protein alpha (*Abpa*) gene on chromosome 7 (Dod et al. 2005); and 6–11) the differences in electrophoretic mobility at 6 neutral or nearly neutral allozyme loci: isocitrate dehydrogenase-1 (*Idh1*, chromosome 1), glucose dehydrogenase-1 (*Gpd1*, chromosome 4), esterase-1 (*Es1*, chromosome 8), mannose phosphate isomerase (*Mpi*, chromosome 9), nucleoside phosphorylase (*Np*, chromosome 14), and superoxide dismutase-1 (*Sod1*, chromosome 16) following the protocols by Bonhomme et al. (1984) and Munclinger et al. (2002).

A genome-wide screen using 485 simple sequence length polymorphisms (SSLPs or microsatellites) was used to estimate the genetic polymorphism preserved in inbred mice. The SSLP loci represent a panel of markers preselected to cover polymorphism between the *domesticus* C57BL/6J strain and the *musculus*-derived PWD strain (Gregorová and Forejt 2000) and are evenly distributed across all autosomes and the X chromosome (Dietrich et al. 1996). One female from each strain was scored at generation ranging from F_{15} (STUS from Studenec; Table 1) to F_{25} (STUP, Studenec; Table 1). One male per strain at the same generation as its female counterpart was scored for one locus located on the Y chromosome, the *Zfy2* intron microsatellite (Boissinot and Boursot 1997). In order to compare the extent of polymorphism of SSLP markers in the new inbred strains developed in this study with similar mouse strains, one male and one female each from the PWD strain derived from wild, pure *M. m. musculus* mice (Gregorová and Forejt 2000) and 3 classical laboratory inbred strains: A/J, C3H/HeJ, and C57BL/6 obtained from a local provider (VELAZ) were also scored.

The majority of SSLPs (450 out of 475 markers) were scored from PCR products on agarose gels. PCRs were performed on a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany) in a 10-μl reaction volume containing 10 mM Tris–HCl (pH 8.5), 50 mM $NH_4(SO_4)_2$, 2 mM

MgCl$_2$ (Fermentas, Burlington, Canada), 0.2 mM of each deoxynucleoside triphosphate (Fermentas), 0.33 μM of each unlabeled primer (Invitrogen, Carlsbad, CA), 0.5 unit of *Taq* polymerase (Fermentas), deionized water, and 30 ng genomic DNA. Each PCR was initiated with an activation step at 94 °C for 2 min, followed by 39 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 60 s, with a final extension of 60 °C for 5 min. Electrophoresis was carried out on 4% agarose gels containing ethidium bromide (10 μg per 10 ml of gel) in tris-borate-edta buffer.

Twenty-five SSLP markers were analyzed using fragment analysis. One of the 2 primers of each locus was 5′-end labeled with a fluorescent dye HEX, 6FAM, VIC, PET, or NED (Applied Biosystems, Foster City, CA), and the loci were amplified in 6 different multiplex PCRs. For each individual and each of the 6 multiplex reactions, PCRs were performed on a Mastercycler ep gradient S (Eppendorf) in a total volume of 10 μl using the Qiagen Multiplex PCR Kit following the manufacturer's protocol and 30 cycles with an annealing temperature of 55 °C for 90 s. The 0.8 μl of PCR products were mixed with 0.25 μl of 500LIZ or 400ROX Size Standard (Applied Biosystems) and 12 μl of formamide (Applied Biosystems) and separated using an ABI PRISM 3130 sequencer (Applied Biosystems). Genotypes were scored using GeneMapper v3.7 (Applied Biosystems).

Phenotype Scores

Immediately after cervical dislocation, for each genetically analyzed individual, body mass and body and tail length were measured. For each male, the onset of maturity (defined as age in days of males with the first occurrence of sperm in their epididymis), sperm count in adult individuals (mean sperm count more than 10 cells of the Bürker hematocytometer in the left epididymis of males aged more than 60 days), mass of left and right testis, and mass of left epididymis were recorded. The breeding performance of each strain was estimated from litter sizes averaged from the previous 6 captive generations.

Behavioral Analyses

In order to demonstrate the utility of the genetic differentiation selected for in our strains, sexual preferences of 60-day-old males and females from F$_{15}$ of 2 of the strains BULS (*musculus* derived) and STRA (*domesticus* derived; Table 1) were scored in a simple 2-way choice system with pools of homosubspecific and heterosubspecific urine (for details, see Bímová et al. 2005). To minimize the role of inbreeding avoidance that might lead to preferences to more different odor (the heterosubspecific one in our case) (Penn 2002), we used urine from strains BUSNA (*musculus* derived) and STLT (*domesticus* derived; Table 1) as signals. Tested animals (20 females and 20 males each from BULS and STRA) were bred in standard conditions as described above. Males were housed individually for at least 5 days before testing, and females were housed with a female littermate. Homosubspecific

preference was estimated as the coefficient of preference R_{signal} calculated from the time spent by sniffing both signals as follows:

$$R_{signal} = (\text{Time sniffing homosubspecific} - \text{heterosubspecific signal})/(\text{Time sniffing homosubspecific} + \text{heterosubspecific signal}).$$

The sign of the coefficient indicates the direction of the preference with positive values indicating homosubspecific preferences. A Student's *t*-test was applied to analyze the deviation of R_{signal} from zero within each strain (H_0: μ = 0), and analysis of variance (ANOVA) was used to test the difference between strains.

Aggressive behavior was studied in 20 F$_{13-14}$ males from each of the 2 strains BULS and STRA (Table 1) following Roubertoux et al. (2005). Each male, housed alone for 5 days preceding the first experiment, was tested repeatedly in 2 experimental dyadic encounters either against a *musculus* or against a *domesticus* male from the same strains. Each dyad consisted of 2 tests with opponents from the same strain: first, the neutral cage arena test and 10 days later followed by the resident–intruder test. The dyads were separated by 30-day pause, and the order of dyads (the origin of the first opponent) was random. Each male was thus tested 4 times against 4 different males at the age of 70, 80, 110, and 120 days. The result of each encounter was classified into 3 categories: winner (the tested male won the encounter), no fight (no aggressive interaction during the 6-min encounter), and loser (the tested male lost the encounter). The χ^2 statistics was applied to identify behavioral differences between the strains, and the significance level was adjusted with a Bonferroni correction.

Immunological Measures

In order to further demonstrate the genetic variation represented by these strains, 5 males and 5 females from F_{14-15} of each of the 2 strains BULS and STRA (Table 1) were used to detect in vitro immunoresponsiveness against 2 antigens: T-cell mitogen Concanavaline A (ConA, Sigma, St. Louis, MO) and B-cell mitogen bacterial lipopolysaccharide (LPS, Difco Laboratories, Detroit, MI). Spleen cell suspensions and macrophages obtained by washing the peritoneal cavity of individual mice were cultivated and measured for 3 types of response: 1) cell proliferation after stimulation with 0.37 or 1.50 μg/ml of ConA and LPS; 2) cytokine production of interleukins IL-2, IL-4, IL-10, or interferon-γ (IFN-γ) in splenic cells and interleukins IL-6, IL-10, or IL-1β in macrophages; and 3) production of nitric oxide (NO) in macrophages. The presence of cytokines in supernatants was measured using an enzyme-linked immunosorbent assay using sets of cytokine-specific capture and detection monoclonal antibodies purchased from PharMingen (San Diego, CA), as previously described by Holáň et al. (1996). Nitrite concentrations in the supernatants were measured using the Griess reaction (Green et al. 1982) and quantified by spectrophotometry at 540 nm using sodium

Table 2. Frequencies of *musculus* alleles at diagnostic loci in wild populations from which mouse strains were collected. Numbers of individuals scored in source populations are indicated in parentheses

Subspecies	Locality	mtDNA	Zfy-2	Tsx	Btk	Abpa	
musculus	Studenec	(18) 1.000	(10) 1.000	(16) 1.000	(23) 1.000	(18) 1.000	
musculus	Buškovice	(62) 1.000	(58) 1.000	(136) 1.000	(137) 1.000	(111) 1.000	
domesticus	Straas	(115) 0.009	(65) 0.000	(147) 0.000	(157) 0.004	(133) 0.000	
Subspecies	Locality	Idh1	Gpd1	Es1	Mpi	Np	Sod1
musculus	Studenec	(11) 1.000	(11) 1.000	(10) 1.000	(11) 1.000	(11) 1.000	(10) 1.000
musculus	Buškovice	(17) 1.000	(17) 1.000	(17) 0.941	(17) 1.000	(17) 1.000	(17) 1.000
domesticus	Straas	(68) 0.088	(67) 0.000	(64) 0.000	(67) 0.000	(67) 0.000	(67) 0.000

nitrite as a standard. Wilcoxon/Kruskal–Wallis nonparametric tests based on rank sums were used to identify among-strain differences in immunological responses. A procedure implemented in JMP statistical software (SAS Institute Inc. 2002) was used to analyze the data.

Results

Genetic Composition of Source Populations of House Mouse Strains

In total, 8 inbred strains were developed by us. Before defining these strains, the genetic composition of source populations based on diagnostic markers is presented to attest to the purity of their genomes.

One source population, from the village of Studenec, is situated within the range of *M. m. musculus* about 250 km east of the center of the Czech portion of the *M. m. musculus/ M. m. domesticus* hybrid zone, and, as expected, all diagnostic markers in the source population were of the *musculus* type (Table 2). The 2 other source populations, one representing wild *M. m. domesticus* and the other the *M. m. musculus* genome, were sampled about 50 km on either side of the center of the hybrid zone in the villages of Straas and Buškovice, respectively (Figure 1). The results listed in Table 2 show that only 1 out of 11 loci in Buškovice (*Es1*) and 3 out of 11 loci in Straas (*mtDNA*, *Btk*, and *IdhI*) were able to introgress across the contact zone, and even these introgressing alleles are found at very low frequencies (maximum 0.088). In addition, some of the alleles that are identical in 2 source populations will be ancestral polymorphisms; therefore, the actual rate of introgression is probably even lower. Hence, the mouse strains derived from these source populations will subsequently be considered as pure *musculus* or *domesticus* strains.

Basic Mouse Strain Characteristics

STUF (Studenec fertile, *M. m. musculus* origin). The first generation of descendants born in captivity segregated for sterility when crossed with C57BL/10: out of 31 mice tested, 14 were sterile and 17 were fertile (Vyskočilová et al. 2005). Full fertility of hybrid males was established after F_5.

STUS (Studenec sterile, *M. m. musculus* origin). All progeny delivered by the founding pair generated sterile males in either type of cross with the C57BL/10 mice

(Vyskočilová et al. 2005). No changes in hybrid male sterility were observed in higher generations (F_5–F_{10}). It has been shown that *musculus* interstrain hybrids (STUF × STUS) can be produced and backcrossed to the C57BL/10 mice to map chromosomal regions harboring genes correlated with sterility (Vyskočilová M, Piálek J, unpublished data).

STUP (Studenec delayed puberty, *M. m. musculus* origin). The progeny of this strain segregated for sterility/fertility until F_{13} when mated with the C57BL/10 mice. Only fertile males have been produced in crosses with the strain C57BL/6 after generation F_{19}. The hybrids between STUP and C57BL/6 have a delayed onset of spermatogenesis (Piálek J, unpublished data).

BULS (Buškovice low sperm, *M. m. musculus* origin). Males of this strain have a low number of sperm in the epididymis. In addition, the strain has the lowest mean litter size (3.26 young per litter) in comparison with other strains described (>4 young per litter).

BUSNA (Buškovice nonaggressive, *M. m. musculus* origin). Since F_{15}, only descendants of males that did not attack the males of the A/J strain were crossed in higher generations.

STRA (Straas aggressive, *M. m. domesticus* origin). Since F_{15}, only males that attacked males of the A/J strain were crossed in higher generations.

STRB (Straas black, *M. m. domesticus* origin). Mice of both sexes have a pronounced black stripe on their dorsal side stretching from the neck to the base of the tail.

STLT (Straas long tail, *M. m. domesticus* origin). This strain and STRB were founded using the same female, but the descendants of either strain were sired by different wild males (Table 1). This is the only strain having a mean tail length greater than body length.

Genetic Polymorphism

Out of 485 SSLP scored, 447 uniquely located and 3 syntenic markers (92.8%) produced PCR products of the expected size under the uniform set of PCR conditions employed. Four primer pairs of SSLP markers (*D10Mit12*, *D11MIt230*, *D15Mit60*, and *D17Mit228*) only amplified alleles of *M. m. domesticus* individuals, whereas 82 markers spread more or less randomly throughout the whole genome only produced bands in some strains.

Fragment analysis of the 25 labeled SSLPs showed that there was only residual polymorphism within the strains

Table 3. Pairwise genetic differentiation based on 361 SSLP markers for the 8 new inbred strains described in this paper. Absolute numbers of different alleles at SSLP loci are above the diagonal and their percentage equivalents are below the diagonal. Gray shaded regions indicate genetic differentiation between the *musculus* and *domesticus* genomes in strains derived from these genomes

	STUF	STUP	STUS	BUSNA	BULS	STRA	STRB	STLT
STUF	—	179	180	202	207	304	305	312
STUP	49.6%	—	188	212	218	287	296	298
STUS	49.9%	52.1%	—	182	204	293	294	295
BUSNA	56.0%	58.7%	50.4%	—	130	295	296	302
BULS	57.3%	60.4%	56.5%	36.0%	—	293	299	300
STRA	84.2%	79.5%	81.2%	81.7%	81.2%	—	137	94
STRB	84.5%	82.0%	81.4%	82.0%	82.8%	38.0%	—	120
STLT	86.4%	82.5%	81.7%	83.7%	83.1%	26.0%	33.2%	

with the highest observed heterozygosity ranging from $H_o = 0.06$ in strains from Buškovice to $H_o = 0.00$ in strains sampled in Studenec. The mean heterozygosity observed in all strains was $H_o = 0.02$.

We compared the genetic differentiation at 361 SSLP loci yielding PCR products in all developed strains (Table 3). Their distribution covers all chromosomes and is regularly spaced (Figure 2). The mean level of microsatellite polymorphism between 2 groups of inbred strains derived from different subspecies is 82%, whereas genetic differentiation between *M. m. musculus* strains from Studenec (STUF, STUP, and STUS) and Buškovice (BULS and BUSNA), respectively, is lower, reaching 57% on average. Genetic polymorphism among the strains derived from wild *M. m. domesticus* from Straas (STRA, STRB, and STLT) is 33% and among those strains derived from wild *M. m. musculus* from Studenec and Buškovice is 51% and 36%, respectively.

Detailed information on all SSLP markers is given in Supplementary Tables and is also publicly available at http://www.studenec.ivb.cz/projects/inbred_strains (Supplementary Tables 1–4). At this web site, it is possible to submit a query for any pairwise combination of inbred strains in order to retrieve the informative markers for a cross or to download a spreadsheet with the locus names, map positions, and relative allele sizes for each marker for all 12 strains scored. The position of SSLP and allele sizes for the 3 laboratory strains, A/J, C3H/J, and C57BL/6J, given in Supplementary Tables 1–4, were retrieved from http://www.informatics.jax.org/.

Morphological Differences

Table 4 presents basic descriptive morphological characteristics for individual strains and their comparison among sexes. Inspection of the table indicates that males were either larger than or equal to females with the exception of BULS (ANOVA, $F_{1,75} = 4.25$, $P = 0.043$). The sexual dimorphism for body mass remains true in groups of strains derived from populations from Studenec (STUF, STUP, and STUS; ANOVA, $F_{1,158} = 6.64$, $P < 0.011$) and Straas (STRA, STRB, and STLT; $F_{1,153} = 26.27$, $P < 0.0001$). For strains from Buškovice (BULS and BUSNA) and all other traits, differences between sexes were not significant.

Post hoc comparisons for strains grouped by locality and sex using a Tukey–Kramer honestly significant difference (HSD) indicated that the *domesticus* strains were both heavier and longer than the *musculus* strains (Straas = C57BL/6 > Buškovice = Studenec, with mean values for body mass of 24.8 = 25.4 > 20.7 = 19.6 g, respectively; ANOVA: $F_{3,466} = 66.60$, $P < 0.0001$; and body length: 92.7 = 91.6 > 87.1 = 86.2 mm, respectively; ANOVA: $F_{3,474} = 61.28$, $P < 0.0001$). All groups differed in tail length (Straas > C57BL/6 > Buškovice > Studenec, with mean values of 91.6 > 83.4 > 69.8 = 67.1 mm, respectively; ANOVA: $F_{3,459} = 473.58$, $P < 0.0001$).

There was also considerable variation in reproductive parameters among the strains (Table 5). The mean litter size was the lowest in strains from Buškovice, significantly higher in strains from Studenec, and the highest in the *domesticus* strains (Buškovice < Studenec < Straas = C57BL/6, with values 3.4 < 5.1 < 6.7 = 7.5, respectively; ANOVA: $F_{3,382} = 88.5$, $P < 0.0001$; pairwise significance tested using Tukey–Kramer HSD comparison). Overall, the *domesticus*-derived strains (from Straas and C57BL/6; mean litter size: 7.3) had larger litter sizes than the *musculus*-derived strains (from Studenec and Buškovice; mean litter size: 4.0; ANOVA: $F_{1,384} = 191.5$, $P < 0.0001$).

A high level of variation was also observed in the mass of male reproductive organs (Table 5). The highest within-locality range was observed for mean testis mass [(left testis + right testis)/2], with males from the STUS strain occupying the lowest rank (0.058 g), whereas that of the STUF males were nearly 50% larger (0.115 g; Table 5). A Tukey–Kramer HSD comparison among localities showed that the *domesticus*-derived males possessed, on average, heavier testes (C57BL/6 = Straas ≥ Studenec > Buškovice, with values for mean testis mass being 0.0908, 0.0908, 0.0813, and 0.0679 g, respectively; ANOVA: $F_{3,286} = 24.4$, $P < 0.0001$) and a heavier left epididymis (C57BL/6 > Straas > Studenec > Buškovice, with values 0.0317, 0.0293, 0.0240, and 0.0195 g, respectively; ANOVA: $F_{3,284} = 163.2$, $P < 0.0001$). The same trend was observed for mean sperm count (Straas = C57BL/6 > C57BL/6 = Studenec > Buškovice, with values 54.1, 50.8, 45.8, and 35.7 sperm per Bürker hematocytometer cell for each group; ANOVA: $F_{3,285} = 42.1$, $P < 0.0001$). Both mean testis mass and left
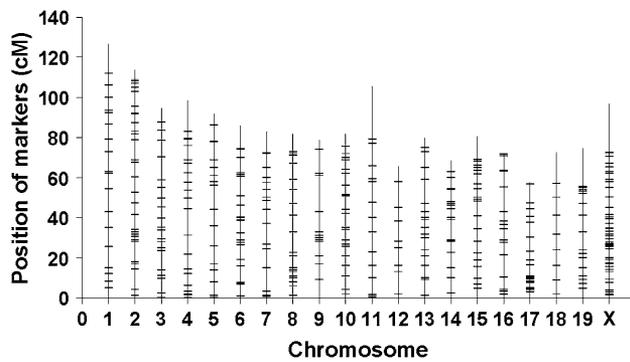
Figure 2. Relative distribution of SSLP markers amplified in all mouse strains except the *Zfy2* intron microsatellite on the Y chromosome. Position of markers (relative to the centromere located at the bottom of each chromosome) is according to Mouse Genome Informatics (http://www.informatics.jax.org/).

epididymis mass were highly correlated with mean sperm count ($r^2 = 0.45$; $F_{1,287} = 235.6$, $P < 0.0001$ for testis mass and sperm count and $r^2 = 0.53$; $F_{1,285} = 329.5$, $P < 0.0001$ for epididymis mass and sperm count). A smaller, but still significant, positive correlation was found between mean sperm count and mean litter size sired by a male ($r^2 = 0.19$; $F_{1,149} = 34.4$, $P < 0.0001$).

Sexual Preferences

The *musculus*-derived BULS males and females displayed strong significant homosubspecific preferences (*t*-test, $P < 0.05$; Figure 3), in contrast to the *domesticus*-derived STRA animals (*t*-test, $P > 0.05$; Figure 3). In addition, the comparison between these 2 strains tested separately for both sexes showed that the differences in assortative mating preference were significant (ANOVA, females: $F_{1,40} = 7258$, $P = 0.01$; males: $F_{1,41} = 24 362$, $P < 0.001$).

Male Aggression

Dyadic encounters resulted in highly significant differences in aggressiveness between the *musculus*-derived BULS and *domesticus*-derived STRA strains (χ^2 test, $P < 0.001$ for both neutral arena and resident intruder test; Figure 4). The aggressiveness increased with asymmetry of resource value with residents being more aggressive irrespective of the strain. The STRA males were aggressive in all tests with both types of opponents (Figure 4) and always won over BULS males. Conversely, the less aggressive males of the BULS strain elicited fights only rarely and only as the owners of their territory.

Immunological Responses

We found significant differences in BULS and STRA strains in response to in vitro stimulation by ConA and LPS. Proliferation of spleen cells differed significantly ($P < 0.001$) between the 2 strains in 2 out of 4 treatments, with

Table 4. Morphological characteristics of wild-derived strains. In each entry, number of individuals scored is followed by mean values of corresponding traits ± standard deviations

Subspecies	Strain	Body weight (g) Males	Females	Body length (mm) Males	Females	Tail length (mm) Males	Females	Mean age (days) Males	Females	Number of individuals Males	Females
musculus	STUF	22.3 ± 2.2 = 21.9 ± 2.5		88.3 ± 2.9 = 90.0 ± 3.9		72.8 ± 3.4 = 74.5 ± 3.0		144 ± 80	139 ± 61	28	21
musculus	STUP	20.2 ± 1.9 > 18.9 ± 1.4		85.6 ± 2.1 = 86.5 ± 3.1		67.1 ± 5.1 = 68.7 ± 5.1		146 ± 83	146 ± 65	30	17
musculus	STUS	18.3 ± 2.3 > 16.9 ± 2.5		84.0 ± 3.6 = 84.3 ± 4.5		59.9 ± 4.7 = 61.4 ± 5.0		190 ± 117	211 ± 90	32	17
musculus	BUSNA	18.4 ± 2.8 = 18.3 ± 2.9		83.8 ± 4.1 = 85.5 ± 5.8		67.8 ± 7.3 = 71.3 ± 7.8		171 ± 70	133 ± 63	23	19
musculus	BULS	21.7 ± 2.3 < 23.0 ± 3.1		88.3 ± 3.0 = 88.9 ± 4.8		70.7 ± 6.9 = 68.5 ± 6.5		181 ± 61	188 ± 63	47	22
domesticus	STRA	27.5 ± 2.3 = 26.1 ± 6.0		94.7 ± 2.8 = 94.1 ± 7.6		93.5 ± 3.8 = 93.1 ± 5.1		140 ± 47	102 ± 52	59	24
domesticus	STRB	24.4 ± 1.6 > 20.3 ± 5.0		92.9 ± 2.1 > 88.6 ± 8.0		89.3 ± 3.5 > 84.9 ± 5.9		152 ± 69	126 ± 79	20	20
domesticus	STLT	23.7 ± 1.5 > 20.3 ± 5.5		93.0 ± 2.2 > 88.8 ± 6.9		95.6 ± 2.7 > 90.5 ± 4.3		150 ± 89	142 ± 70	15	16
"domesticus"	C57BL/6	26.2 ± 3.1 > 23.6 ± 4.2		91.6 ± 3.3 = 91.8 ± 6.1		83.7 ± 4.1 = 82.9 ± 5.5		162 ± 99	169 ± 96	26	10

Within strains differences at significance level $\alpha = 0.05$: =, not significant; >, males larger than females; <, males smaller than females.

Table 5. Reproductive parameters of males. Mean age of adult males is as in Table 4 except data for sperm onset in epididymis, where the first number indicates the number of males investigated from the age of 25 to 60 days. Absolute sperm count per epididymis can be calculated using the formula: (mean sperm count × 2000)/0.0025. Numbers of scored individuals are in brackets

Species	Strain	Mean testis mass (g)[a]	Left epididymis mass (g)	Onset of sperm in epididymis (days)	Mean sperm count	Litter size
musculus	STUF	(28) 0.1154 ± 0.0102	(28) 0.0262 ± 0.0025	(8) 33	(28) 47.3 ± 5.6	(29) 4.38 ± 2.23
musculus	STUP	(30) 0.0749 ± 0.0098	(30) 0.0228 ± 0.0021	(10) 36	(30) 52.1 ± 8.9	(29) 5.62 ± 1.74
musculus	STUS	(33) 0.0582 ± 0.0085	(33) 0.0233 ± 0.0033	(8) 38	(33) 38.8 ± 7.4	(37) 5.22 ± 1.62
musculus	BUSNA	(23) 0.0871 ± 0.0133	(23) 0.0225 ± 0.0048	(3) 32	(22) 54.0 ± 15.6	(35) 4.31 ± 2.01
musculus	BULS	(55) 0.0599 ± 0.0107	(28) 0.0183 ± 0.0024	(6) 33	(55) 28.4 ± 8.0	(149) 3.26 ± 1.65
domesticus	STRA	(60) 0.0975 ± 0.0088	(59) 0.0302 ± 0.0024	(18) 33	(60) 54.2 ± 7.8	(48) 8.83 ± 1.69
domesticus	STRB	(20) 0.0754 ± 0.0051	(20) 0.0274 ± 0.0023	(7) 34	(20) 53.2 ± 7.2	(17) 5.76 ± 1.99
domesticus	STLT	(15) 0.0843 ± 0.0047	(15) 0.0283 ± 0.0023	(12) 35	(15) 55.1 ± 5.4	(15) 5.33 ± 1.95
"*domesticus*"	C57BL/6	(26) 0.0908 ± 0.0089	(26) 0.0317 ± 0.0045	(12) 36	(26) 50.8 ± 9.6	(24) 6.75 ± 1.96

[a] Mean testis mass = (left testis + right testis)/2.

the response being stronger in BULS than in STRA in both cases. Similarly, there were clear differences between strain in production of cytokines (such as NO and interleukins IL-6, IL-10, IL-2, and IL-1β) by peritoneal macrophages stimulated by LPS, with 3 out of 5 tests significant at $P < 0.02$. The same held true for the production of IFN-γ and interleukins IL-4 and IL-10 in spleen cells in response to the ConA treatment (significant differences in 2 out of 3 tests, $P < 0.002$). In 3 out of 5 cases, we detected significantly stronger production of cytokines in BULS than in STRA.

Discussion

Eight new inbred strains have been created from wild populations of both subspecies of the house mouse. Because mice are widely used in biomedical research, these new strains represent a valuable addition to the sources of genetic and morphological variation available from mouse stocks (Bonhomme and Guénet 1996; Festing 1996). In addition, the exact geographic location of founding individuals is known (Table 1), as is the level of genetic polymorphism in source populations (Table 2; Macholán et al. 2007), allowing the rate and effects of genetic reduction due to inbreeding to be estimated (Vyskočilová M, Piálek J, unpublished data). This information, supplemented by complete knowledge of the genetic pedigree of strains, greatly improves the usefulness of these strains as a genetic resource compared with that of laboratory mice, whose unspecified origin hampers the interpolation of studies related to evolutionary processes (Payseur and Hoekstra 2005; Boursot and Belkhir 2006; Harr 2006; Payseur and Place 2007).

The motive for developing these inbred mice was to obtain highly characterized strains that can be potentially exploited to identify barriers preventing gene flow between house mouse subspecies. Two prerequisites are necessary for such studies: there must be sufficient genetic polymorphism to allow genetic mapping of quantitative traits

and high interstrain phenotype variation. Our data suggest that we have succeeded in obtaining both.

First, our results show that on average, 298 of the 361 randomly chosen microsatellite markers are fixed for different alleles in our *musculus*- and *domesticus*-derived inbred strains and 136 are fixed between the groups of *musculus* inbred strains derived from Studenec and Buškovice. Given that chromosome length ranges from 1361 (Dietrich et al. 1996) to 1630 cM (Shifman et al. 2006), the mean spacing between markers for the *domesticus* versus *musculus* genomes ranges from 4.6 to 5.5 cM and for the 2 groups of *musculus*-derived strains (Buškovice vs. Studenec) from about 10 to 12 cM. Silver (1995) estimated that for detecting a linkage signal in 40 backcross samples in the house mouse, 86 genetic diagnostic markers are required to ensure a 10-cM (≈20 Mb) swept radius coverage. Because the mean spacing between markers in our set of strains falls well within this limit, the coverage of strain genomes by markers is dense enough for mapping QTLs in strains with different phenotypes.
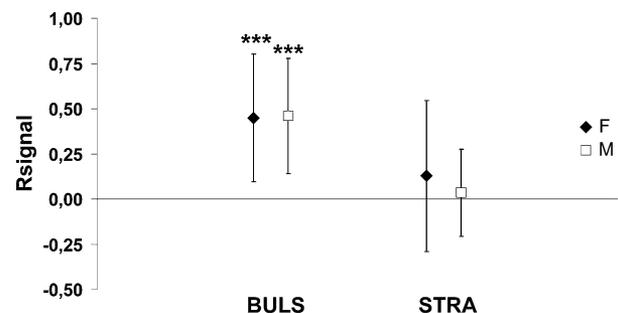
Figure 3. Sexual preferences for urinary signals in *musculus*-derived BULS and *domesticus*-derived STRA inbred strains. Homosubspecific preference is represented by mean $R_{signal} \pm$ standard deviation (see text) and significant values for Student's *t*-test (H_0: μ = 0) are indicated by asterisks; ***$P < 0.005$.
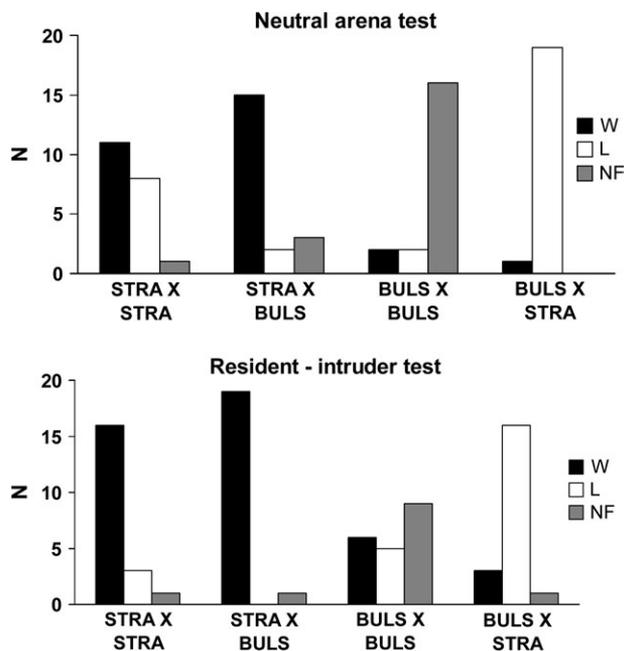
Figure 4. Results of neutral cage arena and resident–intruder test for males of the *musculus*-derived BULS and *domesticus*-derived STRA inbred strains. For each encounter and type of test, the strains of tested and opponent males are listed as tester × opponent. The results of each encounter were classified based on behavior of the tested male and 3 contest outcomes were possible: winner (W), loser (L), or no fight (NF).

This study also documents high interstrain phenotypic variation. In fact, the representatives of *musculus*- and *domesticus*-derived strains (BULS and STRA) have been shown to differ for nearly all morphological traits scored and also in male aggressiveness and assortative mating in both sexes. Moreover, data from in vitro immunological responses suggest that immunoresponsiveness to various mitogens differs substantially between these 2 strains. Although some variation could be attributed to nongenetic factors, the high phenotypic differentiation observed among these strains suggests that significant genetic variation associated with phenotypes has been preserved. This example illustrates the potential usefulness of the genetic differentiation selected for in these strains to evolutionary studies in the *musculus–domesticus* hybrid zone, and further studies are underway to compare more pairs of the strains described here.

The traits contributing to an individual's fitness are of great interest for inferring evolutionary processes. For example, in this regard, one of the most obvious traits is sterility in hybrid males. The 2 *musculus* strains, STUS and STUF, derived from Studenec and producing alternative phenotypes (sterile and fertile offspring, respectively) in crosses with C57BL laboratory mice could be used to search for genetic basis of mechanisms dramatically affecting the fitness of males. In addition, among strains, there is a clear link between reproductive organs of males and their fitness,

with mean testes mass and left epididymis mass positively related to sperm count, which in turn was correlated with mean litter size (see Tables 3–5). Therefore, future experiments using these strains and designed to find QTL correlates of reproductive organ mass in males, and consequently their ability to sire a higher/lower number of offspring, could highlight the processes affecting the dynamics of the hybrid zone.

The ability of an organism to cope with pathogens has a direct effect on its fitness, although this relationship is far from fully understood as a result of its complexity (Lochmiller and Deerenberg 2000; Lee 2006). Our in vitro tests using one representative *musculus*- and one *domesticus*-derived strain indicate clear differences in immune responses to an array of pathogens. This result could reflect subspecies-specific host–pathogen evolutionary histories that may be critical for maintaining the hybrid zone between house mouse subspecies (Sage et al. 1986; Moulia et al. 1991). However, it is not yet clear to what extent immune responses to the mitogens used here reflect resistance to natural pathogens (Goüy de Bellocq et al. 2007). In addition, given the trade-off between immune defense and other fitness components that must share common and limited resources (e.g., reproduction, growth, and development), different strains may also vary in their relative investment in immune defense. Future work will assess the fitness effects of variation in immunocompetence in all these *musculus*- and *domesticus*-derived strains; however, even this single example indicates, once again, the potential usefulness of these strains in evolutionary studies.

Although we have not directly tested the relationship between behavioral traits and fitness components here, there is a wealth of evidence indicating that behavioral patterns are under selection. For example, assortative mating may result in behavioral isolation, representing a strong premating barrier to gene flow and preventing introgression between isolated gene pools (Coyne and Orr 2004 and references therein). In fact, it has been repeatedly shown that the 2 house mouse subspecies are able to discriminate each other and prefer members of their own subspecies and that *M. m. musculus* mice display stronger preferences for homosubspecific signals (Laukaitis et al. 1997; Christophe and Baudoin 1998; Talley et al. 2001; Smadja and Ganem 2002, 2005; Smadja et al. 2004; Bímová et al. 2005). Our results corroborate these studies because irrespective of the sex, the *musculus*-derived BULS mice preferred their own subspecific urinary signal.

Studies relating the level of male aggressiveness in laboratory strains and asymmetry in reproductive output date to the 1970s (Horn 1974; Kuse and DeFries 1976). Asymmetry in the outcome of encounters have also been reported between males from *M. m. musculus* and *M. m. domesticus*, with the latter winning the contests (Thuesen 1977; van Zegeren and van Oortmerssen 1981). More recently, QTL mapping in laboratory mouse strains selected for nonaggressiveness and aggressiveness has suggested a polygenic inheritance of male fighting behavior (Brodkin et al. 2002; Roubertoux et al. 2005). Both the sexual

preferences and the levels of male aggressiveness identified in the *musculus*-derived BULS and *domesticus*-derived STRA strains in this study show that behavioral phenotypes may determine asymmetries in fitness between individuals and strains and, hence, make good candidates for genetic mapping of behavioral isolation. Again, this example demonstrates the potential usefulness of these strains as models for more complex processes occurring in wild populations across the hybrid zone.

In conclusion, this work has resulted in the production of 8 new inbred strains, 3 representing the *M. m. domesticus* and 5 *M. m. musculus* genomes. As demonstrated by the genotyping of 400 microsatellite loci, our inbreeding regime removed nearly all genetic variation from each strain while maintaining high interstrain variation for traits that may be important to the process of speciation; importantly, many of these loci are diagnostic for distinguishing inter- and intrasubspecific strains. In addition, given that controlled breeding conditions were identical for each strain, any interstrain phenotypic differences can be explained by genetic differences, a prerequisite for performing QTL mapping; in fact, 2 strains, the *musculus* strain BULS and the *domesticus* strain STRA were shown here to have significant differences in morphological, behavioral, and immunological traits. Because these phenotypic traits are also correlated with various fitness components, these new house mouse strains potentially represent a powerful tool for inferring the evolutionary processes occurring in the *musculus–domesticus* hybrid zone and the genetics of speciation in general.

Supplementary Material

Supplementary tables can be found at http://www.jhered.oxfordjournals.org/.

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