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Limited variation in the major urinary proteins of laboratory mice

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ABSTRACT

Individual variation in a specialised set of scent communication proteins, the major urinary proteins (MUPs), provides a genetic identity signature that underlies individual and kin recognition, and the assessment of heterozygosity in wild house mice. Here we examine the extent to which MUP variation is retained among 30 classical strains of laboratory mice from three main lineages (Castle, C57, Swiss). Normal wild-type variation in urinary MUP pattern appears to have been lost at an early stage in the derivation of the classical laboratory strains. All strains from the Castle and Swiss lineages shared the same "individual" MUP pattern, consistent with common ancestry from very few founders, while those from the C57 lineage shared a different pattern. Notably, individual variation in MUP pattern was no greater within the Swiss outbred ICR (CD-1) strain than typical for inbred strains. Total urinary protein concentration varied considerably between even closely related substrains, together with minor variation in the relative amount of each MUP isoform expressed, although the functional significance of such quantitative variation in MUP expression has yet to be established. Expression was 2–8 fold higher among males, while a MUP expressed by most male but not female wild mice was expressed by C57 males but variably among Castle and Swiss males and occasionally by females in some strains. The lack of normal variation in MUP patterns within and between strains has important implications for the use of laboratory mice in behavioural or neurophysiological research investigating social recognition or mate choice.

1. Introduction

The urine of house mice, *Mus musculus domesticus*, contains a high concentration of 18–19 kDa proteins (up to mM) of which 99% is made up of a highly polymorphic set of proteins, the major urinary proteins (MUPs) [1,2]. These are a set of species-specific communication proteins [3], encoded by a cluster of at least 19 genes on mouse chromosome 4 [4], that play a number of important roles in scent communication. While some *Mup* genes are expressed in the salivary, lachrymal or mammary glands in mice [5], most mouse MUPs are expressed in the liver and then efficiently filtered into the urine to provide persistent, highly polymorphic signals in mouse urinary scent marks [6,7]. MUP expression is under multihormonal control, with growth hormone, testosterone and thyroxine differentially influencing the expression of different MUP isoforms [8], such that some isoforms are produced predominantly or exclusively by males [4,9] and male wild house mice produce approximately three times the concentration of MUPs overall compared to females [6]. Female MUP concentration increases at oestrus [10], although some studies of laboratory strains have reported extremely low levels of expression in females compared to males [11,12].

MUPs bind volatile pheromones in a central pocket or calyx [13,14], slowing their release from scent marks [15] and helping to concentrate and deliver volatile pheromones to vomeronasal receptors [16,17]. However, other receptors in the vomeronasal organ respond to MUPs themselves when mice make nasal contact with urine scents [18]. In genetically heterogeneous wild mice this highly polymorphic set of proteins provides an individual genetic identity signature that is critical for individual recognition of scent owners [19,20], for kin recognition to avoid inbreeding [21] and for assessment of the genetic heterozygosity of potential mates [22]. Notably, in these contexts the pattern of MUPs in urine scents is used for genetic identity recognition regardless of many other genetic differences that influence an individual's scent, including MHC type. MUPs can also stimulate aggression in male laboratory mice [18], probably due to their central role in social recognition [23].

Despite the importance of MUPs in social recognition and mate assessment, there is limited information available about MUP expression in the many different mouse strains used in research. MUP phenotype should be an important consideration when selecting laboratory strains when similarity or difference in MUP identity signatures could influence test responses or background behaviour. For example, when asking animals to choose between individuals

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with different characteristics, animals need to have different MUP identity signatures to ensure that they can be recognised as different individuals. Most strains used in research are deliberately inbred to be homozygous such that all individuals of the same sex and strain are genetically identical and should share the same MUP type. There are now over 450 established inbred strains, many having been inbred for at least 150 generations [24]. Whether or not different strains have the same individual MUP identity signatures will depend on shared inheritance through the same genetic lineage, as unrelated wild mice each express different MUP patterns [7,25]. The laboratory mouse is not derived from a single wild species but represents a mosaic of genetic material from the two commensal subspecies *M. m. domesticus* and *M. m. musculus*, with a smaller contribution from *M. m. castaneus* [26,27]. Despite earlier widespread beliefs that each inbred strain has a unique genetic background [28], genetic analyses reveal that the classical inbred strains widely used in research all originate from a small founder population [26,27], and possibly even from a single female based on identical mitochondrial DNA sequences [27,29–31]. Many of the most commonly used inbred strains belong to either the Castle or C57 lineages (Fig. 1) and their derivations are well documented [24]. Strains from these two separate lineages exhibit different MUP genotypes and phenotypes [4,32,33], but little is known about the extent to which MUP type varies between strains within each lineage. Further, although lineages may have diverged before the creation of inbred strains, MUP variation between lineages may still be very limited because of their shared origin from the same very limited gene pool. The commonly used Swiss strains of mice, for example, are so-called because they derive from nine non-inbred laboratory mice from Lausanne, Switzerland, but these were previously derived from a colony of laboratory mice of unknown origin at the Pasteur Institute, Paris [34].

Here we examine the extent to which MUP variation has been retained or eliminated in classical strains of mice in three widely used lineages: C57, Castle and Swiss. We also compare variation in inbred strains with a commonly used 'outbred' strain (a closed population that is bred to maintain genetic heterozygosity) to see whether such outbred strains show more variation in their MUP identity signals than inbred strains.

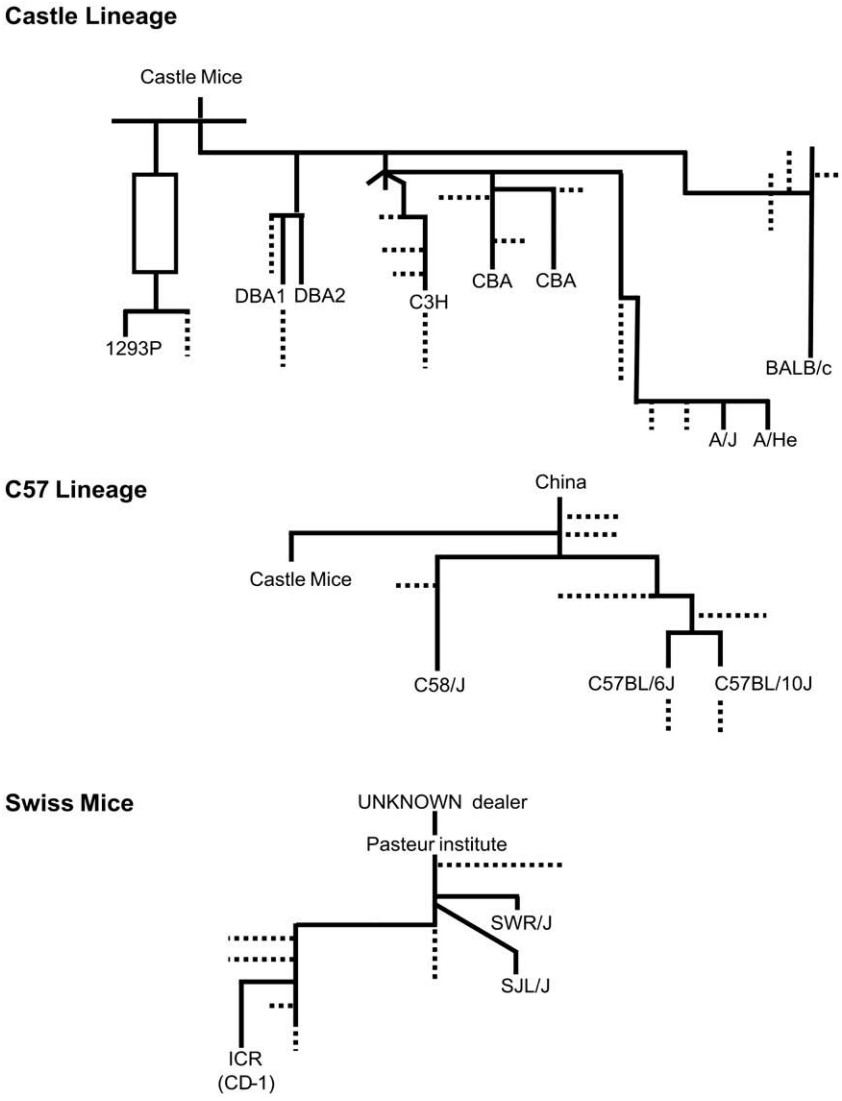

Fig. 1. Origin of inbred mouse strains. The genealogical relationships between individual inbred mouse strains in each of three separate lineages (C57, Castle and Swiss) are simplified from a published version [24].

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2. Methods

2.1. Sample collection

Urine samples were obtained from the Jackson Laboratory (JAX® Mice and Services, 610 Main Street, Bar Harbor, Maine 04609 USA) from five sexually mature individuals (retired breeders) of each sex from 20 different inbred strains from the Castle lineage (A/HeJ, A/J, AKR/J, BALB/cJ, BALB/cByJ, CBA/CaJ, CBA/J, C3HeB/FeJ, C3H/HeJ, C3H/HeSnJ, DBA1/J, DBA2/J, 129P3J), the C57 lineage (C57BL/6J, C57BL/6ByJ, C57BL/10J, C57BL/10SnJ, C58/J) and the Swiss lineage (SWR/J, SJL/J). In addition, six MHC-congenic strains were also sampled (A.BY, A.SW, B10.RIII, B1O.D2/nSnJ, B10.BR, C3H.SW). All mice were housed on pine shavings in polycarbonate duplex cages (33.47×33.47 cm^2), five males on one side and five females of the same strain on the other. Food (standard pasteurised grain) and water (acidified) were provided *ad libitum*.

Additional samples were collected from males of two further inbred strains (C57BL/10ScSnOlaHsd, BALB/cOlaHsd) and their MHC congenic pairs (B10.D2-*H-2*^{d}/nOlaHsd, BALB.K/OlaHsd respectively), together with an outbred strain of Swiss mice (ICR or CD-1). These were obtained from Harlan (Shaw's Farm, Blackthorn, Bicester, Oxon, OX25 1TP, UK). Males were individually housed in North Kent Plastic (NKP) M3 cages (48 cm×15 cm×13 cm) on sawdust with paper wool nest material and access to food (TRM9607, Harlan Teklad) and water *ad libitum*. Wild house mice were caught from farms around Cheshire using Longworth live capture traps containing food (TRM9607, Harlan Teklad), paper wool nest material and either chocolate or peanut butter as bait. Males were housed individually in M3 cages (48 cm×15 cm×13 cm) due to their high levels of aggression, and small groups of females (2–4) were housed in NKP MB1 cages (45 cm×28 cm×13 cm), with peat substrate as bedding and paper wool nest material. They were provided with water and food (TRM9607, Harlan Teklad) *ad libitum*.

2.2. Protein and creatinine assays

As protein concentration varies with the volume of urine eliminated, the ratio of protein to creatinine (both expressed as mg/ml) is routinely measured to correct for urine dilution [6]. Creatinine is a by-product of muscle metabolism and is eliminated almost exclusively in urine. The conversion of creatine to creatinine is a non-enzymic process that proceeds at a constant rate; animals with high muscle mass excrete more creatinine in their urine such that urinary creatinine output is proportional to body mass. For mice with similar body mass, the protein to creatinine ratio thus provides a simple correction for urine dilution.

Protein concentrations were determined using the Coomassie plus® protein assay reagent kit from Perbio Science UK Ltd. (Unit 9, Atley Way, North Nelson Industrial Estate, Cramlington, Northumberland NE231WA). A standard curve was generated from a stock solution of bovine serum albumin (BSA) (1 mg/ml diluted to the range 0–50 µg/ml with ddH_20. Each sample was diluted (typically 1:100 dilution for female samples, 1:500 for male samples), 100 µl aliquots pipetted in duplicate to a 96 well microtitre plate and 250 µl Coomassie reagent added. The absorbance of each sample was read at 595 nm in a Labsystems iEMS-MF plate reader. A standard curve was produced using the Genesis software and the concentration of each unknown sample calculated by interpolation.

Urine creatinine values were measured by the alkaline picrate assay from Sigma Chemicals, UK. A standard curve was generated from a stock solution of creatinine (3 mg/dl diluted to the range 0–30 µg/ml with dd H_20). Each sample (typically 100 µl of 1:100 dilution of urine) was prepared and added in duplicate to a 96 well microtitre plate, together with 150 µl of alkaline picrate reagent (5 ml picrate solution: 1 ml sodium hydroxide). Absorbance at 492 nm was read in a Labsystems iEMS-MF plate reader. A standard curve was produced using the Genesis software and the concentration of each unknown sample calculated by interpolation.

2.3. Gel electrophoresis

Urinary proteins were separated according to their mass using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Urine, clarified by centrifugation at 6700 g for 4 min, was added in a 1:1 ratio to 2× sample buffer (1 M Tris pH6.8, glycerol, 2% SDS, dithiothreitol (DTT), Coomassie Brilliant Blue (CBB)) in a 200 µl capped Eppendorf tube, vortexed to mix, centrifuged for 2 min at 6700 *g* and then boiled for 5 min. The samples were then allowed to cool and centrifuged at 6700 *g* for 2 min before sample loading. Electrophoresis under reducing conditions was at a constant 200 V. Broad range molecular weight markers were purchased from Biorad (Bio-Rad Laboratories Ltd., Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire HP2 7DX) and used in all gels as a comparison. Following electrophoresis, protein bands were visualised using Coomassie Brilliant Blue and destained in a solution of methanol: acetic acid: double distilled (dd) H_2O (30:5:65 v/v/v).

Isoelectric focusing was used to separate urinary proteins according to differences in charge. Proteins were separated on pre-cast, narrow range (pH 4.2–4.9) immobilised pH gradient gels (Pharmacia, UK) rehydrated in 15% glycerol and 2.5% ampholine (pH 3.5–9.5), and run using the Multiphor flatbed electrophoresis system and MultiTemp III thermostatic circulator (Pharmacia, UK). Samples were diluted to 1 mg/ml with ddH_2O and were applied (5 µl) to the gel using sample application pieces (Pharmacia, UK). For the first 200Vh samples were drawn in to the gel. Following this, the sample application pieces were removed and the gel was focused for 14.8 kilo volt hours (KVh) to resolve all proteins according to their isoelectric points. All gels were run at 10 °C. Gels were fixed in 20% (w/v, weight to volume) trichloroacetic acid (TCA) for 1 h; rinsed in a destain solution of methanol: acetic acid: dd H_2O (30:5:65 v/v/v) for 20 min; stained in 0.02% (w/v) CBB with 0.1% (w/v) copper sulphate for 15 min; destained in the above solution for 7.5 h twice, and preserved in 12.5% glycerol (v/v) for 1 h. All development was carried out automatically in the Hoeffer automatic stainer (GE Healthcare,

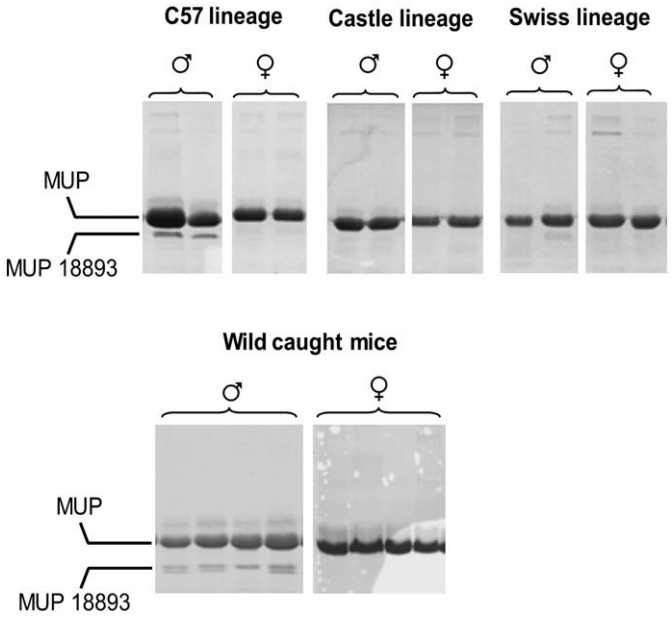

Fig. 2. Pattern of urinary protein expression in three inbred mouse lineages. All urine samples, standardised to a protein concentration of 1 mg/ml, were resolved by 15% 1D SDS–PAGE. Samples representative of each lineage are shown from individuals of the strains C57BL6J (C57 lineage), A/J (Castle lineage) and SWR/J (Swiss lineage). An equivalent analysis of urine from four male and four female wild-caught mice is included for comparison. On each panel, the migration position of most MUPS and the 18,893 Da atypical MUP are highlighted.

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UK). For densitometry, isoelectric focussing gels were scanned in transmission mode and the gel images analysed using the Nonlinear Dynamics (Newcastle Upon Tyne, UK) Total Lab TL100 software to assess the volume of each band.

2.4. Endoproteinase Lys C digestion

Gel plugs (approx. 1 mm^3) were removed from protein bands on the SDS PAGE gel using a thin glass pipette and placed into 200 µl capped Eppendorf tubes. Each gel plug was destained using 100 µl 25 mM Tris HCl pH 8.5, 50% (v/v) acetonitrile and incubated at 37 °C for 30 min. This step was repeated until no stain was visible. The plugs were then washed twice in 100 µl 25 mM Tris HCl pH 8.5, the supernatant discarded and the plugs were incubated with 50 µl 10 mM dithiothreitol. After 30 min at 37 °C the dithiothreitol solution was discarded and 50 µl of freshly prepared 55 mM iodoacetamide stock solution was added to each tube and incubated for 1 h at room temperature in the dark. The iodoacetamide was discarded and the plugs washed twice as above. The plugs were dehydrated in 100% acetonitrile. Following dehydration the plug was rehydrated in 19 µl 25 mM Tris/HCl, 1 mM EDTA, pH 8.5. Sequencing grade Endoproteinase Lys-C (1 uL stock solution (0.1 ug/uL)) was added and the digest was incubated overnight at 37 °C. The reaction was stopped with 1 µl 2.6 M formic acid.

2.5. Peptide mass fingerprinting

The peptides were analysed on a matrix-assisted laser desorption ionisation time of flight mass spectrometer (MALDI-TOF/MS) (Micromass) operated in reflectron mode with positive ion detection. External mass calibration was determined using a mixture of des-Arg bradykinin, neurotesin, ACTH (corticotrophin), and oxidised insulin B chain (2.4, 2.4, 2.6 and 30 pmol/µl respectively) each in 50% acetonitrile and 0.1% (v/v) trifluroacetic acid. Samples were mixed 1:1 (v/v) with a saturated solution of α cyano-4-hydroxycinnamic acid in acetonitrile:water:trifluoracetic acid (50:49:1 v/v/v). Peptide mass fingerprints were searched against the MSDB protein database (ftp://ftp.ebi.ac.uk/pub/databases/MassSpecDB/) using the MASCOT search engine (www.matrixscience.com).

3. Results

3.1. Qualitative differences in urinary MUP expression

First we confirmed by SDS–PAGE that the only major protein bands in the urine of healthy mice correspond to MUPs. As expected, mice of all strains and both sexes exhibited a strong characteristic MUP band migrating at approximately 20–21 kDa that accounted for nearly all of the protein in each sample. A lower intensity band of higher mobility

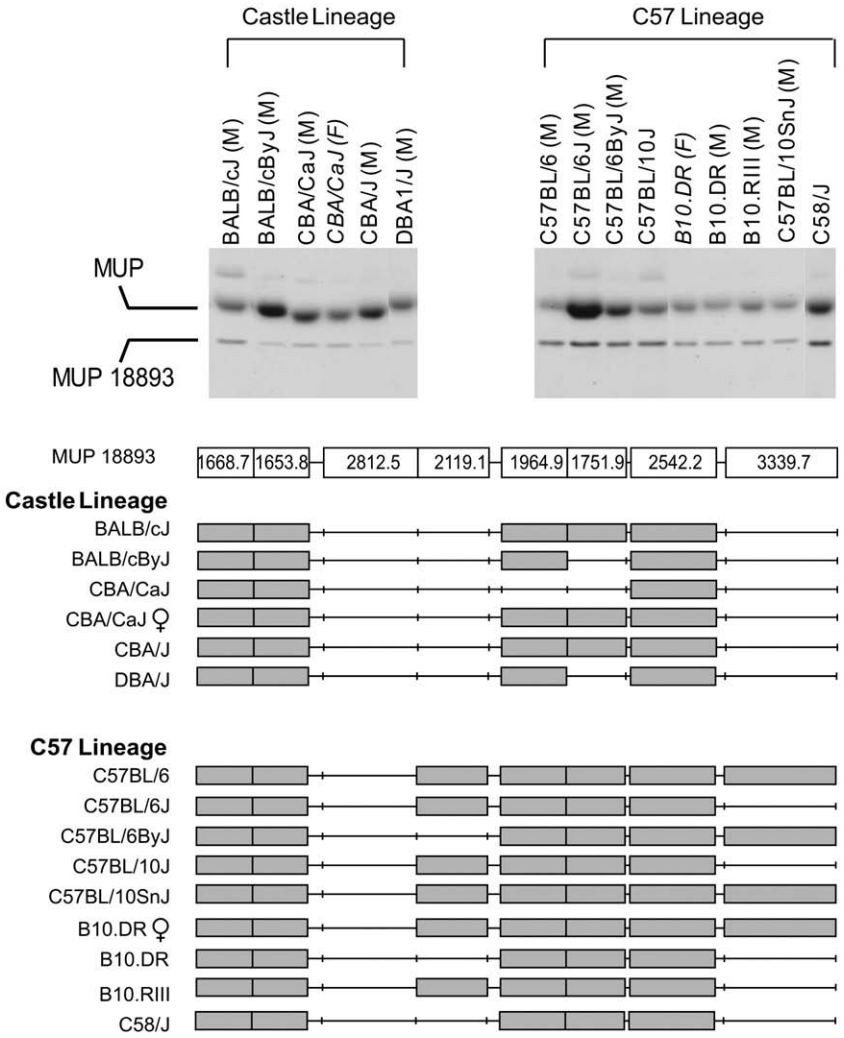

Fig. 3. Expression of the 18,893 Da atypical MUP in inbred mouse strains. Urinary proteins of either sex were resolved on 15% SDS PAGE and the high mobility band (marked as MUP 18893) was analysed by peptide mass fingerprinting by endopeptidase Lys C digestion. The proteolytic peptides were analysed by MALDI ToF mass spectrometry and the peptide masses were related to the cDNA predicted sequence for the 18,893 Da MUP [9]. For each protein band, the peptides matched to 18,893 Da MUP are highlighted as shaded boxes.

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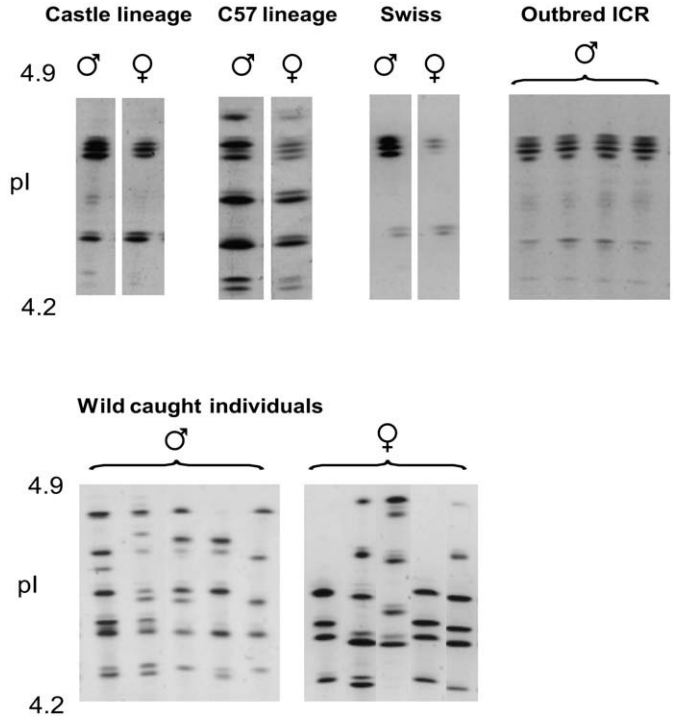

Fig. 4. MUP profile complexity in different lineages. Urine samples, standardised to 1 mg/ml protein concentration, were resolved by isoelectric focusing. Representative patterns are shown for a male and female BALB/cByJ (Castle lineage), C57BL/6J (C57 lineage), SWR/J (Swiss lineage), and for four individual males of the outbred Swiss ICR strain. For comparison, five male and five females from wild caught mice were analysed in the same way.

was also expressed by all males of the C57 lineage (Fig. 2). This band has previously been fully characterised in C57BL/6 male mice as a MUP of 18893 Da [9], encoded by C57BL/6 *Mup* gene 17 [4], that exhibits higher mobility on SDS–PAGE gels than predicted by its size. This MUP exhibits substantial sequence divergence from other urinary MUPs [4], has specific affinity for binding the male pheromone 2-*sec*-butyl 4,5-dihydrothiazole and is known to exhibit male-specific expression in both the C57BL/6 strain and in wild *M. m. domesticus* [9]. Interestingly, although we also observed this band predominantly in males, this was not exclusively the case across all laboratory strains. While males from all eight of the C57 lineage strains examined expressed the high mobility band, a similar band was visible in some females from three of the C57 strains (visible in two out of five C57BL/6J, three out of five B10.DR and three out of five B10.RIII females). By contrast, the band was typically absent from most (68/90) of the male samples from the 16 Castle lineage and two Swiss lineage strains (Fig. 2), but was evident in males from 5/16 Castle strains and in some individual females from 1/2 Swiss and 3/16 Castle lineage strains. Peptide mass fingerprinting confirmed that in each case the high mobility band matched the 18,893 Da MUP from C57BL/6 mice and is thus likely to be the same MUP or one of very similar sequence in all strains (Fig. 3).

We further characterised the pattern of MUPs expressed by each strain and sex by separating MUPs according to their charge using isoelectric focusing (IEF). Samples from wild mice showed a high degree of individual variation as expected (Fig. 4). By contrast, all individuals across seven out of eight C57 lineage strains shared a very similar IEF band pattern while those in 15 out of the 16 Castle lineage strains exhibited a second IEF pattern different from that of the C57 lineage (Fig. 4). This agrees with previous reports that mice from these two separate lineages express two different protein expression patterns [4,32,33] but extends this to a considerably greater range of strains within each lineage. Within each strain, male and female patterns were broadly similar but usually differed in the presence or relative intensity of two or three bands (Fig. 4), corresponding to known sex differences in expression of both the 18,893 Da MUP (above) and another MUP of 18645 Da which are expressed predominantly by males [4]. Any variations in IEF pattern between strains from the same lineage were restricted to the relative intensity of particular shared bands, consistent with quantitative but not qualitative differences in their MUP patterns (though see below for one exception). We also examined two inbred strains (SWR/J, SJL/J) and one outbred strain (ICR or CD-1) of Swiss mice. These Swiss strains shared the same IEF MUP pattern as those from the Castle lineage, strongly suggesting that the Swiss lineage shares common ancestry with the Castle lineage (Fig. 4). Notably, although outbred mouse strains are deliberately bred to maintain genetic heterozygosity within a closed population, variation in MUP pattern between individuals of the ICR(CD-1) outbred strain was no greater than between individuals within each inbred strain. This implies that normal wild-type variation in MUP genotype was lost before this outbred strain was created.

3.2. Quantitative differences in MUP expression

When examining qualitative differences in MUP pattern expression above, gels were deliberately loaded with an equivalent amount of protein from each sample. However, within each strain males excreted more urinary protein than females (comparison of mean±SE

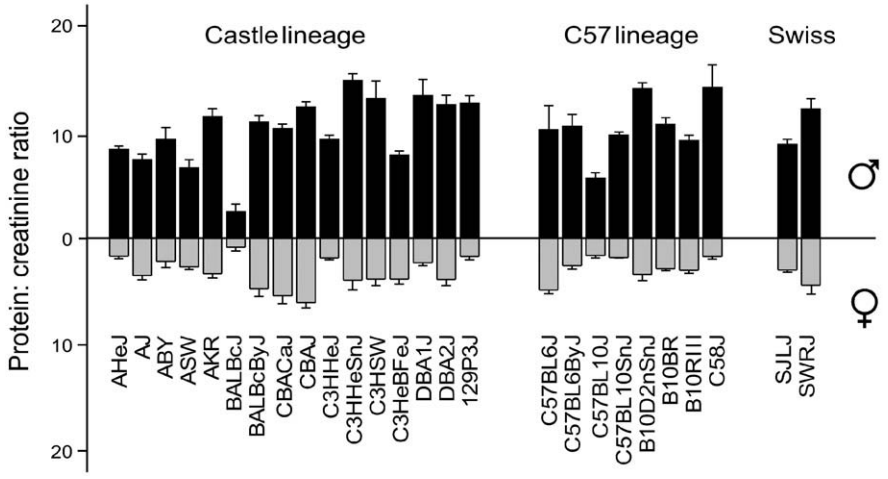

Fig. 5. Protein output in different laboratory mouse strains. Protein and creatinine concentrations were measured for five male and five female urine samples from each strain to generate a dimensionless protein:creatinine ratio that reflects protein output corrected for urine dilution. Males are represented by solid black bars, females by solid grey bars (mean±SE).

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mg/ml protein per strain for males = 13.07 ± 0.71, females = 3.20 ± 0.32; $t = 16.28$, $n = 27$ strains, $P < 0.0001$; corrected for urine dilution as the urinary protein to creatinine ratio per strain for males = 10.26 ± 0.55, females = 3.05 ± 0.25; $t = 14.18$, $n = 27$ strains, $P < 0.0001$), a sex difference which ranged from approximately twice as much protein in males in the two CBA strains up to over eight times as much in males in the C58/J strain (Fig. 5). There were also substantial differences in total urinary protein concentration between strains. This was not due to differences in protein output between the lineages corresponding to the two different MUP patterns seen, but instead reflected substantial differences in total urinary protein between even very closely related substrains from the same lineage (substrains are derived from a common ancestor but have been separated for more than 20 generations in different laboratories). Urinary protein output differed significantly in at least one sex among each of the six pairs of substrains in our study (Table 1). The same was also true for three out of four MHC-congenic strains compared with their parent strain (Table 2). The level of expression exhibited by females across the different strains was very similar to that of genetically heterogeneous wild house mice that were similarly captive bred and housed in small single sex groups (protein creatinine ratios for $n = 27$ females, 3.0 ± 0.3, range 1.1 to 9.4). By contrast, laboratory strain males generally had lower levels of expression compared to captive-bred wild males ($n = 24$, 19.7 ± 1.4, range 7.9 to 33.8), although it should be noted that wild males had to be singly rather than group housed unlike the laboratory males shown in Fig. 5 because of their high levels of aggression. At present, little is known about the effects of different housing conditions on urinary protein levels.

Although detailed comparisons of MUP intensity differences between each strain were beyond the scope of this study, we undertook a quantitative comparison of MUP IEF patterns for males of two commonly used pairs of MHC-congenic strains. Fully congenic strains are expected to be genetically identical at all loci except for the transferred locus and a linked segment of the relevant chromosome. As MHC is on chromosome 17 and the MUP complex is on chromosome 4, MHC-congenic strains should have the same MUP genotype and genetic background, differing only in MHC and closely linked genes. Confirming this, the congenic strain pair C57BL/10ScSnOlaHsd and B10.D2-*H-2*^{d}/nOlaHsd both expressed the same MUP IEF pattern typical of C57 lineage strains, while the congenic strain pair BALB/cOlaHsd and BALB.K/OlaHsd expressed the same MUP IEF pattern typical of Castle lineage strains. However, within each congenic strain pair, there were small but significant quantitative differences in the relative intensities of some of the bands quantified by gel densitometry (Fig. 6). Thus, although there is no reason to expect any difference in MUP genotypes between these congenic pairs, there were still minor differences in the relative intensities with which specific MUP isoforms were expressed.

Table 1
Comparison of urinary protein to creatinine ratios between substrains

		Male			Female		
Strain	Substrain	Mean ± SE	$F(1,8)$*	P	Mean ± SE	$F(1,8)$*	P
A	A/J	7.4 ± 0.5			3.5 ± 0.4		
	A/HeJ	8.4 ± 0.3	2.77	0.135	1.7 ± 0.2	16.42	0.004
BALB	BALB/cJ	2.3 ± 0.6			0.8 ± 0.3		
	BALB/cByJ	10.9 ± 0.6	97.28	<0.001	4.7 ± 0.7	23.60	0.001
CBA	CBA/J	12.3 ± 0.5			6.0 ± 0.5		
	CBA/CaJ	10.3 ± 0.5	8.23	0.021	5.4 ± 0.7	0.56	0.477
C3H	C3H/HeJ	9.3 ± 0.4			1.8 ± 0.2		
	C3H/HeSnJ	14.8 ± 0.7	52.31	<0.001	3.9 ± 0.9	5.26	0.051
C57BL/6	C57BL/6J	12.1 ± 1.6			4.9 ± 0.4		
	C57BL/6ByJ	10.5 ± 1.1	0.01	0.908	2.6 ± 0.3	22.03	0.002
C57BL/10	C57BL/10J	5.2 ± 0.5			1.6 ± 0.3		
	C57BL/10SnJ	8.9 ± 0.1	51.75	<0.001	1.5 ± 0.1	0.04	0.843

*Substrain pairs compared within each sex by ANOVA using SPSS version 14.0.

Table 2
Comparison of urinary protein to creatinine ratios between MHC congenic strains

		Male			Female		
MHC congenic strain pairs		Mean ± SE	$F(1,8)$*	P	Mean ± SE	$F(1,8)$*	P
C3H	C3H/HeSnJ	14.8 ± 0.7			3.9 ± 0.9		
	C3H.SW	13.1 ± 1.7	0.90	0.370	3.8 ± 0.6	16.42	0.545
C57BL/10	C57BL/10SnJ	8.9 ± 0.1			1.5 ± 0.1		
	B10.D2	14.1 ± 0.5	94.46	<0.001	3.4 ± 0.6	11.89	0.009
	C57BL/10SnJ	8.9 ± 0.1			1.5 ± 0.1		
	B10.BR	10.7 ± 0.6	8.42	0.020	2.8 ± 0.2	45.93	<0.001
	C57BL/10SnJ	8.9 ± 0.1			1.5 ± 0.1		
	B10.RIII	9.2 ± 0.5	0.32	0.586	2.8 ± 0.2	24.91	0.001

*MHC congenic strain pairs compared within each sex by ANOVA using SPSS version 14.0.

3.3. Atypical MUP patterns within strains

Genetically identical individuals of the same strain and sex should express the same pattern of MUPs, but we did not find this to be true for all strains examined. Mice of the C58/J strain derive from the same male as other strains of the C57 lineage, although from a different female. Male C58/Js expressed the typical C57 lineage MUP pattern, but females exhibited individual variation in the IEF bands expressed (Fig. 7A). While three females expressed the same pattern as other C57 lineage females, two of these had particularly low concentration of three normally low intensity bands. The other two females of this strain appear to express a completely different pattern not seen in any of the other laboratory strains sampled. The most likely explanation is that a mutation within this strain has affected MUP expression, at least among females, although this has not reached homozygosity across the strain.

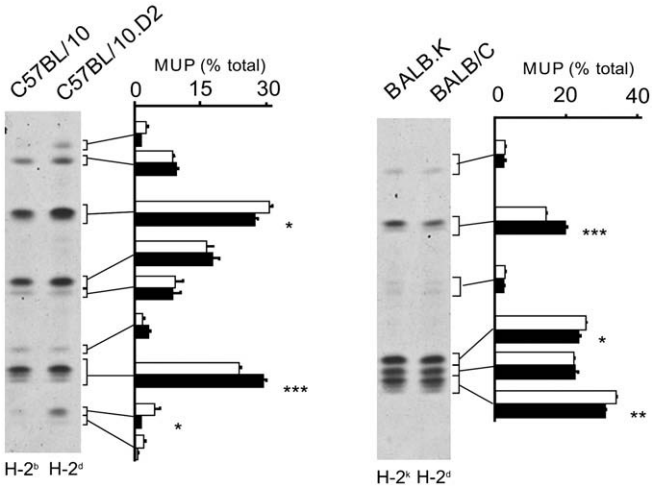

Fig. 6. The effect of MHC haplotype on urinary MUP expression. Urinary MUPs from two strains (BALB/c and C57BL/10) and their MHC congenic counterparts (BALB.K and C57BL/10.D2) were resolved by isoelectric focussing, the MUP isoforms quantified by densitometry and the relative contribution of each isoform determined (mean ± SE, $n = 5$ individuals per strain arranged in congenic pairs across the gel). t-tests compared the relative expression of each protein band between MHC congenic strains (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). Representative expression profiles are aligned to the histograms.

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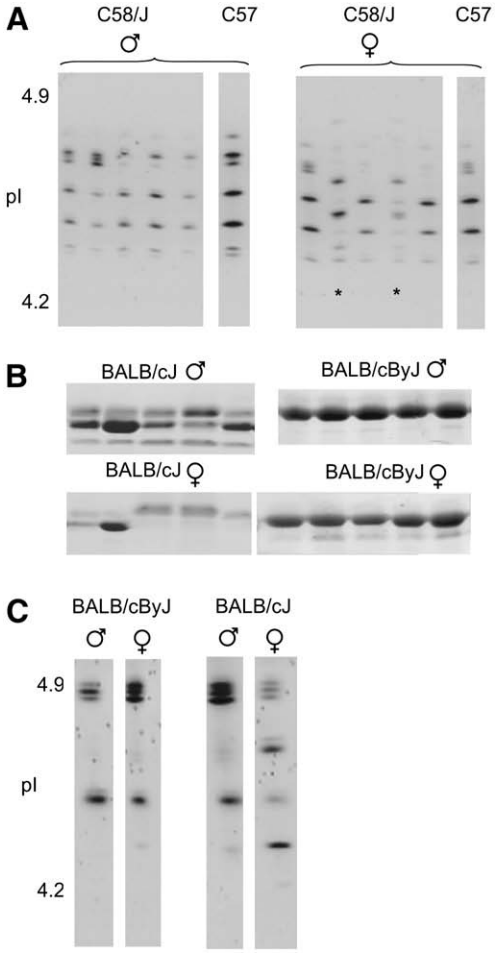

Fig. 7. Atypical MUP expression patterns. Urine samples from both sexes of specific inbred mouse strains (C58/J BALB/cByJ and BALB/cJ) were analysed by isoelectric focussing (panels A, C) and 1D SDS–PAGE (panel B).

One of the sixteen Castle lineage strains also exhibited striking differences compared to closely related substrains. The BALB/cJ strain had much lower urinary protein levels than other strains including the closely related substrain BALB/cByJ (Fig. 5; Table 1). MUPs from the BALB/cJ strain also exhibited an unusual migration pattern on SDS–PAGE gels although the precise pattern varied between individuals (Fig. 7B). Separation by charge revealed further variation in this unusual strain, which exhibited weak expression of bands that partly matched some bands of the typical Castle lineage pattern and some of the C57 lineage pattern (Fig. 7C). The BALB/cJ substrain has been reported previously to express unusually low urinary MUP concentrations as well as exhibiting unusual behaviour, including greater aggression and cannibalism, higher brain weights and lower mean serum testosterone concentration than other substrains of BALB/c mice [35,36].

4. Discussion

Although we examined a large number of mouse strains from three apparently separate genetic lineages, only two basic MUP profiles were apparent, together with some atypical variation within just two of the strains examined. This is in marked contrast to wild house mouse populations (*M. m. domesticus*) where there is considerable variation in the patterns of MUPs expressed between unrelated individuals [25,37,38, present study]. As there are many different MUP haplotypes within wild populations, and outbred animals normally inherit different haplotypes from their mother and father, only wild individuals that are closely related are likely to share the same MUP genotype. Further, MUP heterozygosity is directly maintained in wild mice because they avoid inbreeding with relatives that share both of the same MUP haplotypes as themselves [21]. By contrast, inbred laboratory mice are genetically homozygous and thus express a simpler MUP pattern with fewer MUP isoforms than typical heterozygous wild mice. What is much more remarkable is that variation in MUP patterns is not only lacking within each homozygous inbred strain but also between strains, with the exception of a clear dichotomy between strains from the C57 lineage and those from the Castle and Swiss lineages. This provides further strong evidence that all of the classical or 'old' inbred mouse strains widely used in research are derived from an extremely limited founder gene pool, in agreement with mitochondrial DNA evidence. This restriction in genetic variation may have occurred either during the derivation of laboratory mice at the beginning of the last century or during the early derivation of pet mice approximately 3000 years ago [29], when normal variation in MUP patterns must have been lost. This is consistent with the origin of the Swiss mouse lineage from random-bred laboratory stock at the Pasteur Institute [34], which presumably already carried the MUP pattern typical of all Castle strains. It has been argued that colonies of outbred Swiss mice have retained nearly the same amount and type of genetic variation as that found in natural murine populations [39], but this is clearly not the case for MUP variation. Indeed, given the evidence that all of these mice originate from such limited stock, it is very hard to see how such colonies can resemble the genetic variance typical of normal outbred individual wild mice.

Despite the many generations of laboratory mice that have now been bred across many different strains, there has been very little mutation of MUP haplotypes. Out of a total of 30 mouse strains examined in this study, we found unusual MUP patterns in only two strains. Abnormal MUP expression in the BALB/cJ strain has been reported previously, but it is not clear whether this is due to a mutation in the MUP region or to a mutation that has reduced testosterone production [36], which is one of the main hormones controlling MUP expression among males [8,40]. Individual variation in IEF band patterns among C58/J females may reflect one or more mutations in the MUP isoforms expressed by this strain that has not reached homogeneity. It is unlikely that this variation is due to accidental mixing of the strain with another (at least with another classical inbred strain), as the new bands observed do not correspond with those expressed by Castle or Swiss strains, but further work is needed to investigate this more thoroughly. However, there was some interesting variation between strains in the expression of a male-specific MUP that appears to play a key role in binding one of the most abundant male volatile pheromones [9]. Although this gene is present in strains of both MUP types from the C57 and Castle lineages [4] and is consistently expressed in most male wild mice [9], expression among male Castle and Swiss strains varied. Our finding that some individual females also showed weak expression of this MUP, although this has not been observed among wild females, may be due to relaxation of sexual selection in the laboratory.

The main variation between strains was in the total amount of urinary protein rather than in the pattern of MUPs expressed. As urinary MUP expression is under multi-hormonal control, differences between even closely related substrains may reflect subtle differences in hormone levels. Overall, the mean level of urinary protein expression among laboratory strains was approximately three times lower for each sex than typical for wild-caught mice [6], although the upper range observed among laboratory strains overlapped with levels observed among wild-caught animals. However, this reduction appears to be due to environmental effects, at least in part, as wild females bred in captivity and kept in single sex caged groups had similar low levels of expression to laboratory strain females. Nonetheless, the difference between males and females was similar to that

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of wild-caught mice and we failed to confirm the extremely low levels of expression in females of some strains reported previously [11,12]. This may be because these much earlier studies did not use sufficiently sensitive methods to accurately assess levels in females. So far, evidence for the role of MUPs in scent communication has focused on response to variation in MUP pattern and it is not yet known whether differences in total MUP concentration between strains provides information that might influence their communication. Intriguingly, there were small but consistent differences in the relative intensities of expression of different MUPs between strains with the same MUP pattern, even between MHC congenic strains (evident as differences in the relative intensities of the same band on IEF gels). It is well established that genes within the MHC region influence growth and hormone levels [41] and it is likely that this will also influence the expression of the different MUP isoforms. However, it is doubtful that these small differences have any functional significance for communication. Wild mice use considerably greater qualitative variation in MUP patterns to recognise individuals and kin and to assess potential inbreeding among conspecifics [19–22]. Minor variations in MUP expression due to hormone levels are unlikely to provide a useful genetic identity signal if this can vary with individual condition, but such variation may play a role in communicating information about an individual's current status and needs to be investigated further. Further, as volatile scents associated with MHC type are bound to urinary proteins [42], it is possible that such subtle differences in MUP pattern contribute to the well-established differences in volatile scent profiles between MHC congenic strains [43–45].

The lack of variation in MUP patterns between strains has important implications for a wide range of behavioural research that uses these strains to address questions concerning social recognition or mate choice. MUP variation among wild mice provides an important polymorphic individual genetic identity signal that underlies the ability to recognise and to assess potential mates from their urinary scents [20–22,46], as well as recognition of individual competitors [18,19]. Lack of variation in this key identity signal is thus likely to be highly problematic for research that requires animals to be able to recognise individuals and/or kin using the normal naturally selected recognition mechanisms used in this species. For example, when animals are asked to discriminate between two potential mates, they must be able to easily recognise which animal is which. Even if animals are from apparently “outbred” strains with other genetic differences between individuals, a lack of MUP variation is likely to have a considerable impact on the ability to recognise and assess different individuals even though mice readily detect and investigate other genetic and non-genetic differences in scents that may not signify identity [20,47]. Where experiments attempt to assess response to “unfamiliar“ animals, the stimulus animals should have different, unfamiliar MUP patterns to ensure that they are recognised as unfamiliar individuals rather than animals with a familiar individual genetic identity signal but whose scent has unfamiliar characteristics which might signify changed status. One potential solution is to use ‘new’ strains that have been separately derived from the wild, thus are completely unrelated to the classical strains and should have different MUP types. Even so, within each of these inbred strains animals will share the same MUP type and a range of strains may be needed, balanced across treatment groups.

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