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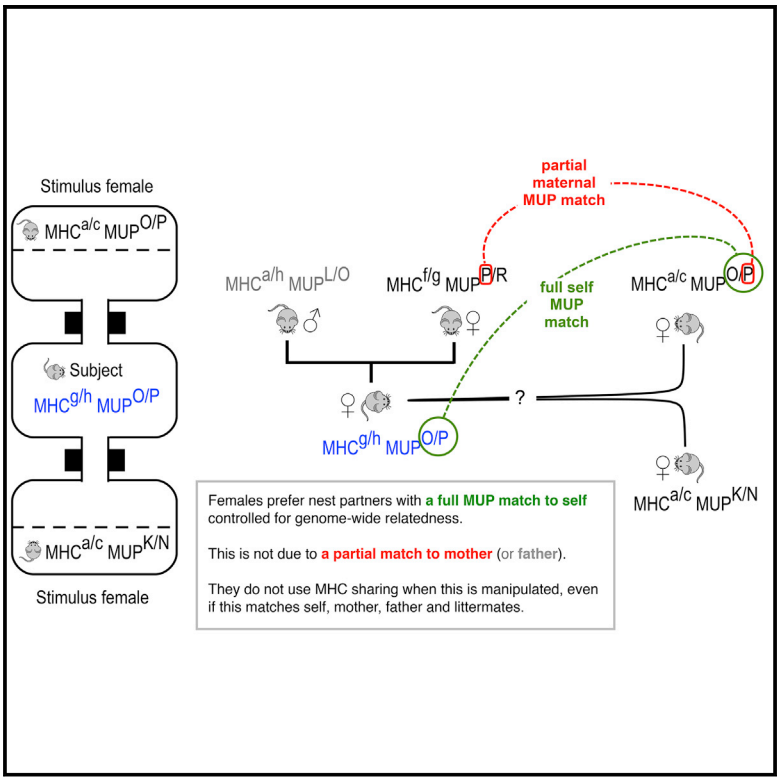
The Genetic Basis of Kin Recognition in a Cooperatively Breeding Mammal

Graphical Abstract



Highlights

- Female house mice use genetic markers to choose closely related nesting partners
- They strongly prefer partners sharing their own major urinary protein (MUP) genotype
- Without MUP sharing, partners sharing multiple loci across the genome are preferred
- MHC sharing is not used; instead, MUP provides a species-specific kinship marker

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In Brief

Female house mice can breed cooperatively and usually select related nest partners, but the genetic markers they use to recognize kin are unknown. Green et al. show that mice prefer partners that match their own major urinary protein (MUP) genotype, a species-specific kinship marker. Contrary to widespread assumption, MHC sharing is not involved.

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The Genetic Basis of Kin Recognition in a Cooperatively Breeding Mammal

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SUMMARY

Cooperation between relatives yields important fitness benefits, but genetic loci that allow recognition of unfamiliar kin have proven elusive. Sharing of kinship markers must correlate strongly with genome-wide similarity, creating a special challenge to identify specific loci used independently of other shared loci. Two highly polymorphic gene complexes, detected through scent, have been implicated in vertebrates: the major histocompatibility complex (MHC), which could be vertebrate wide, and the major urinary protein (MUP) cluster, which is species specific. Here we use a new approach to independently manipulate sharing of putative genetic kin recognition markers, with the animal itself or known family members, while genome-wide relatedness is controlled. This was applied to wild-stock outbred female house mice, which nest socially and often rear offspring cooperatively with preferred nest partners. Females preferred to nest with sisters, regardless of prior familiarity, confirming the use of phenotype matching. Among unfamiliar relatives, females strongly preferred nest partners that shared their own MUP genotype, though not those with only a partial (single-haplotype) MUP match to themselves or known family. In the absence of MUP sharing, females preferred related partners that shared multiple loci across the genome to unrelated females. However, MHC sharing was not used, even when MHC type completely matched their own or that of known relatives. Our study provides empirical evidence that highly polymorphic species-specific kinship markers can evolve where reliable recognition of close relatives is an advantage. This highlights the potential for identifying other genetic kinship markers in cooperative species and calls for better evidence that MHC can play this role.

INTRODUCTION

In cooperatively breeding species, individuals can gain indirect fitness benefits by helping kin to reproduce [1], but reliable mechanisms are needed to distinguish close kin. Discrimination could be achieved by matching phenotypes encoded by highly polymorphic genetic loci in other individuals [2, 3], allowing recognition of relatives carrying genetic markers regardless of prior familiarity. To be useful, though, kinship markers must normally correlate strongly with sharing across the rest of the genome. This creates a special challenge for identification of the specific loci used for kin recognition, as tests of putative kinship markers must fully control for matching at any other loci that could play a role [3–6]. Indeed, among vertebrates, genetic markers used to assess kinship have yet to be definitively identified, particularly in the context of cooperative behavior. However, two highly polymorphic gene complexes have been implicated as putative kinship markers, both of which influence individual scent cues.

Odors associated with the highly polymorphic major histocompatibility complex (MHC) are the textbook example of a putative kinship marker, with the potential to apply across all vertebrates [7, 8]. In fact, evidence is surprisingly scarce from studies that properly control sharing at other loci across the genome. When equally related siblings (sibs) are tested, juvenile arctic char (*Salvelinus alpinus*) prefer waterborne odor from those sharing their MHC IIb genotype [4, 9], and African clawed toad tadpoles (*Xenopus laevis*) preferentially shoal with those of the same MHC genotype [5, 10]. In both cases, though, preference for shared MHC type does not extend to unfamiliar non-sibs [9, 11]. If MHC-based discrimination occurs only between sibs, this would not function as a genetic kinship marker. The main evidence that MHC type directly influences odor-mediated discrimination comes from inbred strains of laboratory mice in which MHC type is the only difference between individuals [12–14]. Some strains of inbred male mice prefer mates of a different MHC type from their mother due to familial imprinting on parents (but not littermates) during rearing [13, 15, 16]. However, this model tests only for discrimination against those genetically identical to a familiar parent (thus, parent recognition). It does not test the crucial requirement that a specific kinship marker is recognized in other genetically distinct

individuals through phenotype matching. To address more naturalistic scenarios, early studies crossed MHC types from laboratory mice onto a semi-wild genetic background to provide heterogeneous animals with a restricted set of MHC haplotypes [17–19]. Correlations in these experiments between MHC sharing and kin-biased behavior (mate selection or communal nursing between females) are consistent with the hypothesis that MHC acts as a genetic marker of kinship. Crucially, though, as in studies of non-model species in natural populations [20], correlations with other loci shared through kinship are not controlled.

Another highly polymorphic cluster of at least 21 functional genes on mouse chromosome 4 encodes the major urinary proteins (MUPs) [21, 22], inherited independently of MHC. These specialized communication proteins are present at high concentration in mouse urine. The patterns of MUP isoforms expressed by genetically heterogeneous house mice (*Mus musculus domesticus*) are used for individual recognition [23–25] and to assess genetic heterozygosity [26]. Like MHC, the MUP region is inherited as a haplotype of tightly linked genes. Mice inheriting the same MUP genotype on heterogeneous backgrounds express similar phenotypes, evident in females (Figure S1) as well as in males [23, 27]. Thus, MUPs also have strong potential for providing a genetic kinship marker in mouse urine. An initial test assessed whether sharing MUP and/or MHC haplotypes influenced mating preferences when background relatedness was controlled among wild-stock mice breeding freely in large semi-natural enclosures [3]. Consistent with use of MUP type as a kinship marker to avoid inbreeding, there was a substantial deficit of mating between those of the same MUP genotype. By contrast, mating was not reduced when male MHC haplotypes matched the female or her mother. However, disassortative mate preferences could also arise from heterozygous advantage at the putative marker itself (for example, improved immunity for MHC and individual and/or heterozygosity signaling for MUP) rather than signifying use of a kinship marker to avoid inbreeding across the genome. Such large-scale naturalistic approaches also provide very limited evidence concerning the mechanisms involved and cannot test the full range of phenotype-matching templates that could be used. This requires the ability to manipulate the specific rearing experiences and genetic inheritance of individual animals while simultaneously controlling for experience of matching at all other loci.

Here we develop a different approach to solve this longstanding problem to establish the genetic markers and recognition templates used for recognition of unfamiliar close kin among normal, genetically heterogeneous animals. Using a carefully designed captive breeding program, we generated family lines of outbred wild-stock house mice. This provided a large selection of unfamiliar individuals with different parents that were all equally related to each other within a family line (coefficient of relatedness, $r = 0.19$ or 0.25) to control for genome-wide sharing. Each individual carried different random combinations of MHC and MUP haplotypes, tracked by descent through family pedigrees; in utero and during rearing, they also experienced different sets of haplotypes from their mothers and littermate sibs for potential familial imprinting. Thus, responses could be tested toward unfamiliar kin that differed in their match to the individual subject at one of the two putative kinship markers, while we controlled for any match at the other marker and across the genome. Different matches could be assessed either to the subject itself or to haplotypes that the subject had experienced during rearing (but did not carry itself) to test for any familial imprinting.

We use this approach to test whether female wild-stock house mice (*Mus musculus domesticus*) use genetic kinship markers based on shared MHC haplotypes, MUP haplotypes, and/or other genes to preferentially establish cooperative associations with related females. House mice live in family-based social groups, but mixing between relatives and nonrelatives is extensive. Females nest socially and often cooperate to rear offspring in communal nests, where each breeding female provides milk and other care to the communal litter [28]. Prior familiarity between females is a major factor influencing the success of communal nests [28]. Communal nursing partnerships are established with nest sharing before females reproduce, with females choosing to share nest sites with preferred partners [29, 30]. In free-ranging environments, females prefer to nest and communally rear offspring with close kin such as sisters [18, 31, 32], but relatedness and prior familiarity are conflated in such studies. Familiar close kin could be recognized using learned individual-specific cues rather than genetic kinship markers, so our first experiment established that females prefer to form nest alliances with close kin (sisters) over unrelated females using genetic kinship markers, regardless of prior familiarity. We then tested the specific genetic markers and recognition templates that they use.

RESULTS

Recognition of Kinship Does Not Require Familiarity

To test partner preferences with genetic sharing and prior familiarity manipulated independently, we gave wild-stock female house mice a choice between a sister and an unrelated female. Stimulus animals were either (1) both unfamiliar (a non-littermate sister versus age-matched nonrelative from other cages) or (2) both familiar cagemates (littermate sisters cohabiting from conception were housed with age-matched nonrelatives from weaning to reflect the mixing of unrelated animals once independent). Thus, females could recognize familiar sisters through individual-specific cues learned during rearing and by phenotype matching of genetic markers, but they could use only phenotype matching of genetic markers to recognize unfamiliar sisters. We established an assay in which each subject female could move freely between two potential nest partners or a neutral cage to assess the independent preferences of subject females (Figure 1A). Nest partner preferences were assessed over a 72 hr test period to ensure that choices were consistent over time and reflected a real preference for nesting with another female rather than simple investigation of scents.

Females showed a strong preference to spend time with a sister versus an equivalent age-matched nonrelative ($p = 0.001$) and, overall, prior familiarity had no influence on preference for kin ($p = 0.48$; Figure 1B). To check that this association preference reflected a choice to nest with a sister, we broke down behavior into the inactive light and active dark phases of the light cycle. This confirmed that in the light, when females were largely inactive and resting, there was strong preference to nest with a

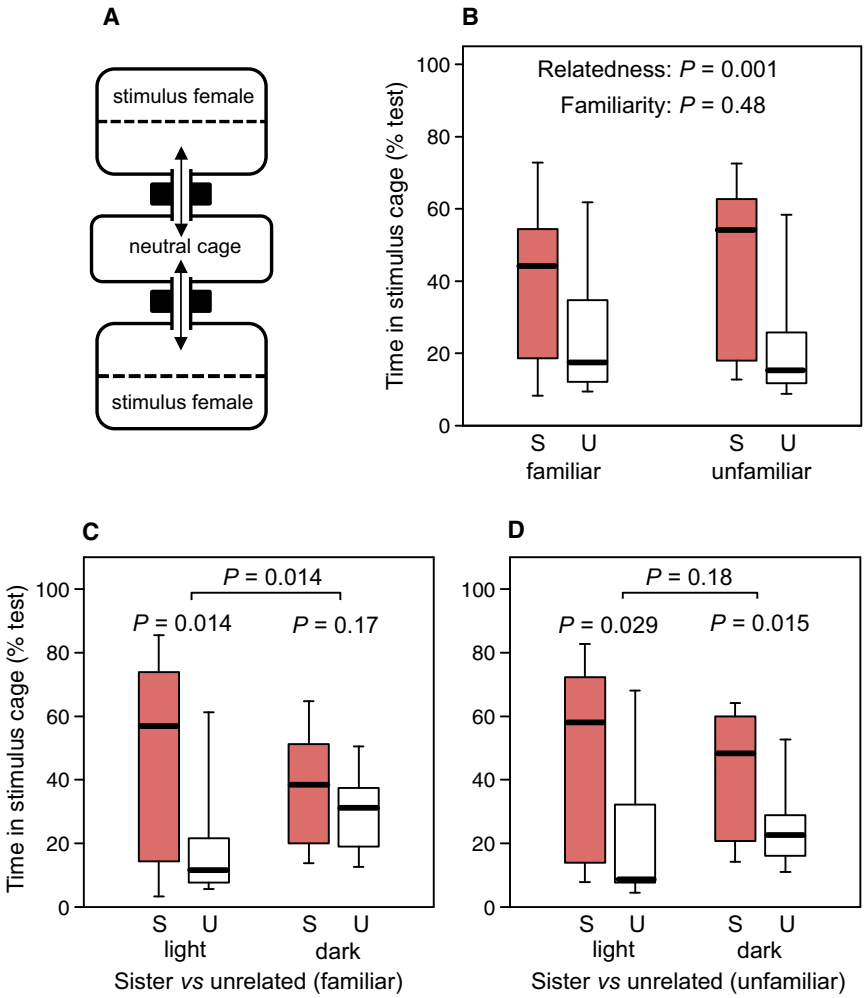

Figure 1. Females Prefer to Associate with Sisters over Unrelated Females

(A) Subject females could move between cages housing a sister, an unrelated female or a neutral cage, with their direction of movement through the linking tunnels being monitored continuously (black boxes).

(B–D) Percentage of total trial time (72 hr) in the sister (S, red fill) versus unrelated (U, open) female stimulus cage when both were either familiar cagemates (n = 19) or unfamiliar (n = 22) to the subject (B). Boxes show median and interquartile range with 10% and 90% whiskers. A linear mixed model, taking additional random factors into account including age and weight differences, confirmed a highly significant preference for sisters and no difference according to previous familiarity (Table 2). Time in stimulus cages is broken down into the inactive light phase, when females nested together, and the active dark phase for familiar cagemates (C) and unfamiliar females (D). Wilcoxon matched-pair tests within and between light phases confirmed that females preferred to nest with sisters during the light phase, whether previously familiar with the females or not.

sister, whether familiar or unfamiliar (Figures 1C and 1D). In the active dark phase, preference to actively interact with an unfamiliar sister remained strong, though this bias reduced when both females were highly familiar cagemates (Figures 1C and 1D). This clear recognition of sisters as preferred nest partners, even when previously unfamiliar, indicates that kin bias is based on a process of phenotype matching rather than individual recognition of familiar relatives [33]. This could be based on recognition of shared genetic markers and/or other cues gained from similarities in maternal environment.

Kinship Recognition Is Based on MUP, but Not MHC, Loci

To establish whether females use MHC and/or MUP haplotypes to recognize close kin as preferred nest partners, we assessed a female's preference between two unfamiliar age-matched relatives (coefficient of relatedness both r = 0.19 or both r = 0.25). These differed in their match to the subject at one or both of the two putative markers and derived from different parents from the subject. Very tight linkage of genes within the MHC and MUP clusters allowed sharing of complex haplotypes to be tracked very reliably within family pedigrees through recent common descent, with animals from the same family line sharing one haplotype (partial match), both haplotypes (full match), or none (no match) at each putative marker (Figure 2A). Microsatellite markers spread across each region checked for any recombination events, but these were rare (0.2% of MHC and 0.7% of MUP haplotypes inherited; see the Supplemental Experimental Procedures and Figures S2 and S3). We also found tight linkage between MHC and the cluster of 38 *Esp* genes that encode exocrine-gland-secreting peptide (ESP) pheromones involved in mouse olfactory signaling [34] (see the Supplemental Experimental Procedures). Thus, any effects due to MHC sharing could potentially be explained by differences in MHC and/or ESP type. Figures 2B–2E and Table 1 provide illustrative examples of the matching versus non-matching stimulus female genotypes selected for each type of test, according to both a subject's own genotype and a subject's parental genotypes.

Full Self Match at MHC or MUP

First, we tested whether females preferred unfamiliar partners that fully matched themselves (both haplotypes shared) at either MHC or MUP when sharing was controlled across the rest of the genome, including the other marker (Figure 2B; Table 1, test 1). Females strongly preferred to associate with partners that shared their own MUP type over those that shared no MUP haplotype through common descent ($p = 0.001$; Figure 3A). Preference to nest with a MUP-matching partner was evident during the inactive light period, in addition to more time being spent with the matching partner during the active dark period (Figure 3C). By contrast, there was no preference for partners that fully matched the female's own MHC type over those that shared no MHC haplotype (Figure 3B),

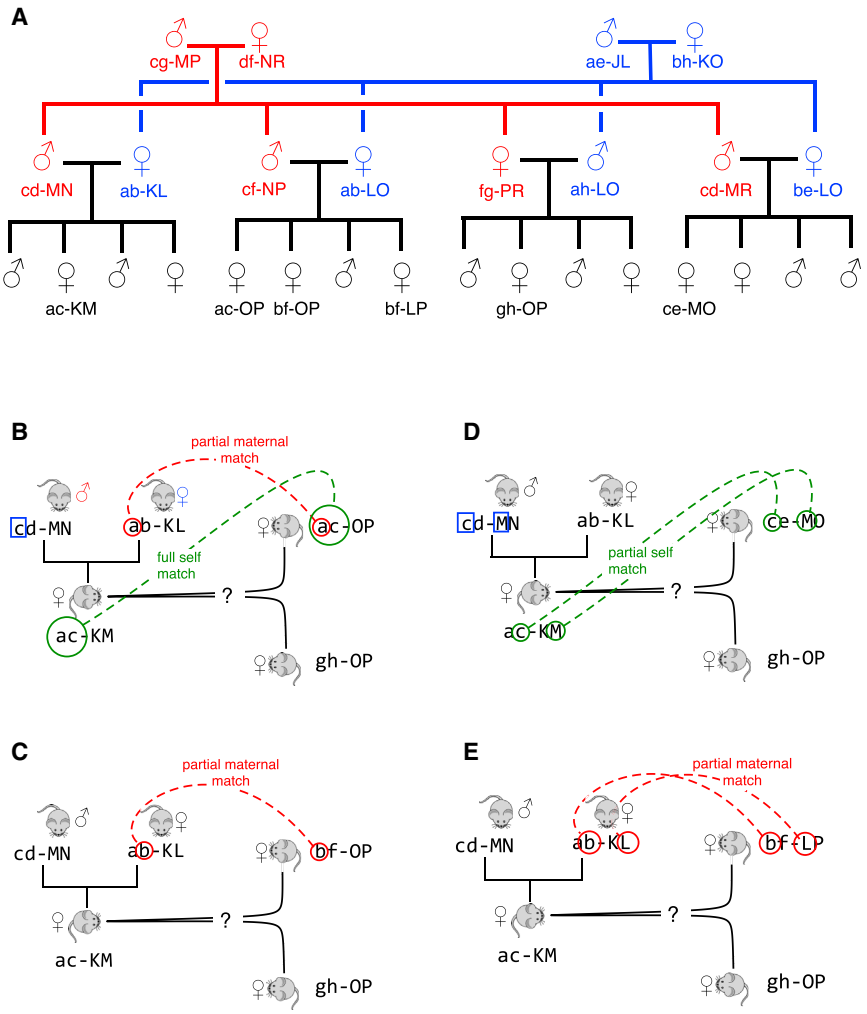

Figure 2. Testing Matches at MHC and/or MUP while Controlling for Genome-wide Sharing

(A) Family lines were created by breeding two unrelated families of outbred sibs (red, blue) that were then crossed to provide multiple litters of unfamiliar double cousins (r = 0.25) as illustrated (black) or a set of double cousins was then crossed with an unrelated set of sibs to provide multiple litters related at r = 0.19 (not shown). Within each line, litters were equally related but had different parents and family experience of MHC (lower case) and MUP (upper case) haplotypes (see also Figures S2 and S3).

(B–E) Examples of matching and non-matching stimulus animals used to assess nest partner preference according to their match to a subject female (highlighted in green) and her familial exposure (red, maternal; blue boxes, paternal, though sires themselves were not present during rearing). Stimulus females were equally related and unfamiliar to the subject. Shown are full self match and partial maternal/paternal match at MHC (B), partial maternal but no self match at MHC (C), partial self/paternal match at MHC and MUP (D), and partial maternal but no self match at MHC and MUP (E). Table 1 provides full list of test types with example genotypes.

a lack of preference that persisted through both active and inactive periods.

Haplotype Imprinting

Recognition of partners that match their own MUP type could be achieved by self-referent matching [35], but could also be achieved by imprinting on cues from relatives learned during development. Offspring can imprint on odors of the animals they are reared with [14, 19, 36], particularly on those from their mother, with which they share one allele at every locus and are exposed to intimately in utero and throughout lactation. As partners that fully match the subject's MUP type carry one MUP haplotype that is also familiar through a partial (single MUP haplotype) match to the subject's mother and to other offspring in the nest (Figure 2B), preference could be due to a match to themselves and/or to a partial match to their mother and other sibs. To distinguish between these mechanisms, we tested whether females preferred unfamiliar partners that shared a haplotype with the subject's mother and littermates, but not with themselves (potentially learned through familial imprinting), over an equally related female that carried two novel haplotypes at the focal marker that they had not experienced during rearing (Figure 2C; Table 1, pooled responses to test 2, 3, or 6 where the haplotypes carried by the non-matching female were both novel).

Females failed to associate preferentially with partners that matched a highly familiar maternal MUP haplotype (Figure 3D). Thus, preference for a full match to their own MUP type was not due to familial imprinting during rearing. There was also no evidence for familial imprinting on maternal MHC haplotypes experienced during rearing (Figure 3E). Indeed, unexpectedly, the effect of MHC on partner preference was opposite to that predicted by the hypothesis that females could recognize kin based on a 50% match (one shared haplotype) to their familiar mother's MHC type [19]. From a theoretical viewpoint, it is unclear why this might be, and further studies will be needed to establish whether this apparent opposite bias has any functional significance. Here, we focused only on identifying the shared genetic markers that females use to preferentially associate with kin.

Preference for a full MUP match to themselves, but not for a single-haplotype match to their mother and littermates, might be because females use only self-referent matching. Alternatively, they may recognize a full match to any MUP phenotypes learned from themselves or imprinted from known relatives. To test this, we asked whether females prefer partners that fully match their mother's MUP type over equivalently related females that do not (Table 1, test 3). Preference for a full match to maternal MUP type was not significant (Figure 3F). This contrasts with the consistent preference when partners matched their own MUP type (Figure 3A), suggesting that imprinting on other familiar MUP types experienced during rearing does not have a strong effect on partner preference. Neither was there any indication of preference for partners that shared the subject's

Table 1. Example Subject and Stimulus Trios Used for Each Test of Kin Recognition

Test	Subject Type[a]	Dam Type	Stimulus	
			Matching	Non-matching
1. Full Self Match (and Partial Maternal Match)				
Marker 1 (focal)	ac	ab	ac	gh
Marker 2	KM	KL	OP	OP
Relatedness			0.19/0.25	0.19/0.25
2. Partial Maternal Match				
Marker 1 (focal)	ac	ab	bf	gh
Marker 2	KM	KL	OP	OP
Relatedness			0.19/0.25	0.19/0.25
3. Full Maternal Match (and Partial Self Match)				
Marker 1 (focal)	ac	ab	ab	gh
Marker 2	KM	KL	OP	OP
Relatedness			0.25	0.25
4. Partial Self/Paternal Match				
Marker 1 (focal)	ac	ab	ce	gh
Marker 2	KM	KL	OP	OP
Relatedness			0.19/0.25	0.19/0.25
5. Partial Self/Paternal Match, Both Markers				
Marker 1 (focal)	ac	ab	ce	gh
Marker 2 (focal)	KM	KL	MO	OP
Relatedness			0.19/0.25	0.19/0.25
6. Partial Maternal Match, Both Markers				
Marker 1 (focal)	ac	ab	bf	gh
Marker 2 (focal)	KM	KL	LP	OP
Relatedness			0.19/0.25	0.19/0.25
7. Genetic Background				
Marker 1	ac	ab	ef	ij
Marker 2	KM	KL	OP	TV
Relatedness (focal)			0.19	0

Arbitrary example of haplotypes carried by one subject female at two genetic markers (upper- or lowercase) inherited from unrelated heterozygous parents. Prior to testing, subjects had experience of all haplotypes carried by themselves, their mothers, and their littermates (sire not present during rearing).

[a]Separate tests (1–7) were based on haplotype matching at the focal genetic marker(s) (MHC, MUP, and background), where matching was to the subject itself and/or to the subject's mother (matching haplotypes are underlined). In tests 2 and 6, the matching stimulus shared a haplotype with the subject's mother and some littermates, but not with the subject. In tests 4 and 5, the matching stimulus shared a haplotype with the subject and some littermates (paternally derived), but not with the subject's mother. Stimulus animals were of equivalent relatedness (either r = 0.19 or r = 0.25), except in test 7. For tests 1–4, MHC or MUP acted as the focal marker in separate tests, with matching at the other marker controlled (either no match, as in the example, or haplotypes were equivalently matched by both stimulus animals). In all tests, there was no sharing between subjects and the nonmatching stimulus at the focal marker. For test 7, MUP and MHC haplotypes of both stimulus animals were unfamiliar to the subject prior to testing (i.e., not shared with the subject, the subject's parents, or the subject's littermates).

full maternal MHC type (Figure 3G). The sample size for this test was small (n = 13) due to the limited availability of appropriate unfamiliar stimulus females, but the direction of response was opposite to that predicted by a kinship marker.

Lack of Preference for Single-Haplotype Matching

As females preferentially associated with those sharing a full MUP match to themselves, we asked whether they also prefer partners that share just one of their two MUP haplotypes. Unrelated animals are very unlikely to share both haplotypes at a highly polymorphic gene cluster, providing reliable exclusion of non-kin. However, this supports recognition of only a proportion of close relatives (approximately one-third of full sibs in an outbred population with eight different haplotypes [37]). Many more close relatives share a single polymorphic haplotype, but this is also considerably more likely between non-kin too (approximately half will share a single haplotype in a population with eight different haplotypes [37]). Thus, use of single-haplotype sharing would allow more relatives to be recognized but at the cost of much less reliable exclusion of non-kin. This high risk of mistaken association with matching non-kin could be halved if a single haplotype had to be matched at both MUP and MHC [37].

To assess the effect of single-haplotype matching to themselves on nest partner preference, we tested matching at the female's paternally inherited haplotype, as the father was not present in the nest during rearing (Table 1, tests 4 and 5; note that recognition of the maternally inherited haplotype was tested in the full maternal match model, test 3). Females displayed no preference at all for partners that matched their paternally inherited MUP haplotype (Figure 3H) or that matched their paternally inherited MHC haplotype (Figure 3I). Instead, they tended to spend more time with an equally related female with no match. Sharing a single haplotype at both markers simultaneously (Figure 2D; Table 1, test 5) did not significantly improve discrimination based on each marker alone (Figure 3J; Table 2). Thus, full sharing with themselves at the MUP marker influences nest partner preference between female house mice, but not partial sharing. Full sharing is a conservative mechanism that reliably excludes non-kin as preferred partners and is likely to reflect very close kinship, even though only a limited proportion of close kin will be recognized using this mechanism.

The high risk that many non-kin will share a single haplotype at a single kinship marker is the same whether self-referent or maternal comparison is used to recognize kin. Thus, we also tested whether females use a single maternal haplotype match at both MHC and MUP to reduce this risk through a maternal imprinting mechanism (Table 1, tests 2 and 6); this template would allow recognition of all maternal sibs because they inherit a maternal haplotype at both putative markers. However, there was no evidence that sharing a single maternal MUP haplotype and a maternal MHC haplotype influenced nest partner preference (Figures 3K–3M; Table 2).

Although it has been suggested that mice might recognize a large proportion of close relatives by recognizing separate maternal MHC haplotypes inherited by other kin [19], currently there is no evidence that mice can perform such single-haplotype matching in other individuals at either MHC or MUP. Such recognition mechanisms may be limited by constraints on the ability to resolve complex polymorphic phenotypes into the

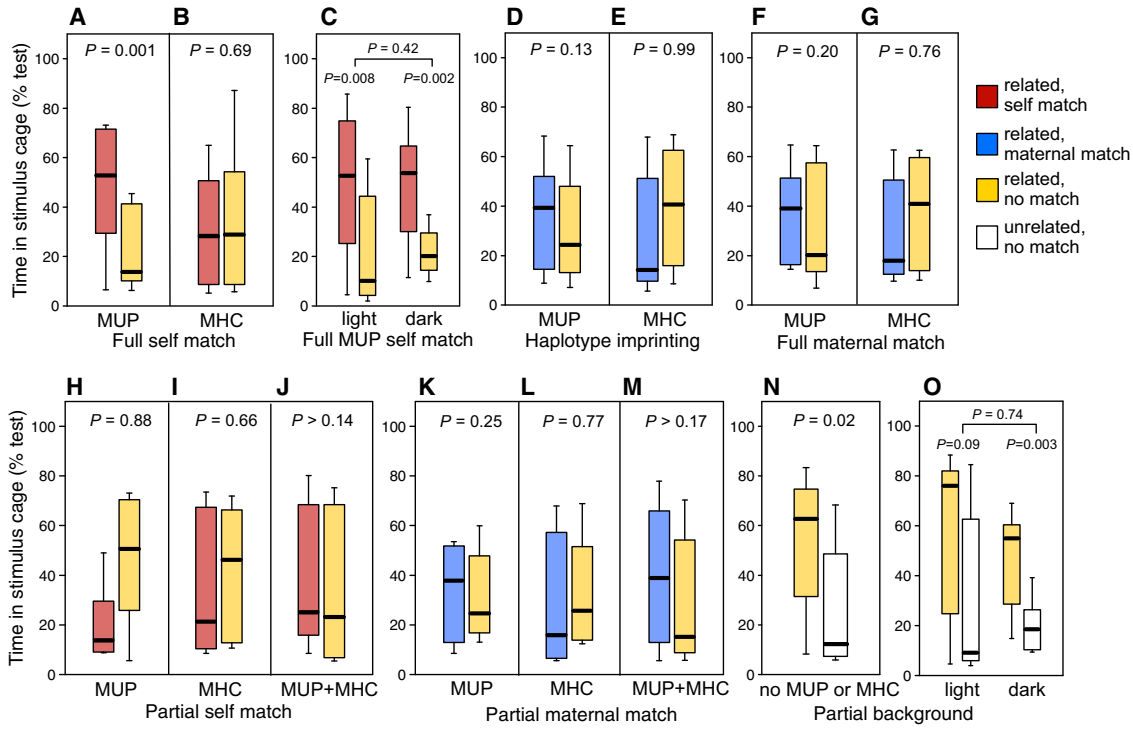

Figure 3. The Influence of Different Genetic Markers on Nest Partner Preference

Nest partner preference (Figure 1A) was assessed over 36 hr between unfamiliar females that were either of the same relatedness (both r = 0.19 or 0.25) but differed in match at MUP and/or MHC (A–M) or differed in relatedness (r = 0.19 versus 0) but shared no MUP or MHC with the subject or subject's mother or littermates (N and O). Full match indicates both haplotypes; partial match indicates one haplotype, shared with the subject itself (red bars in A–C and H–J) or only with its mother and littermates (blue bars in D–G and K–M). The matching stimulus for haplotype imprinting (D and E) carried a haplotype familiar from the subject's mother and littermates at the focal marker but not shared with itself, while neither haplotype of the non-matching stimulus had been experienced during rearing. Boxes show median and interquartile range with 10% and 90% whiskers (n sizes: A, 19; B, 19; C, 19; D, 35; E, 33; F, 19; G, 13; H, 14; I, 12; J, 16; K, 10; L, 11; M, 16; N, 16; and O, 16). p values from linear mixed models assess preference for the female that matches the relevant genetic marker (A, B, and D–N; Table 2). For genetic markers in which there was significant preference, time in stimulus cages is broken down into the inactive light phase, when females nested together, and the active dark phase (C, full MUP match to itself; O, background relatedness but no MUP or MHC haplotypes shared or familiar). Wilcoxon matched-pair tests within and between light phases confirmed that bias was similar during light and dark phases, though nesting together was more variable for shared relatedness but no MUP or MHC haplotypes (O). No preference for nesting with a matching female during the light phase was evident in tests of other genetic markers.

contributions of separate haplotypes, particularly when this must also be achieved on varying genetic backgrounds. Further, to provide the same reliable exclusion of non-kin that can be achieved by full sharing with themselves at a single highly polymorphic locus, single-haplotype matching would require assessment across multiple independent polymorphic markers [37] and would not be achieved by partial matching at MUP and MHC markers alone.

Other Genetic Loci Contribute to Kinship Recognition

Attention has focused on odors associated with MHC and MUP types as potential kinship markers because of very high levels of polymorphism at these loci, together with proven influence on individual scent. However, many genes influence individually variable scents in mice [38, 39]. We asked whether females use matching at other genetic loci to recognize unfamiliar relatives when no MUP or MHC haplotypes are shared (either through common inheritance or experienced during rearing). Females were tested with an unfamiliar relative from different parents (r = 0.19) versus an age-matched nonrelative (matched female from an unrelated family line), when neither had any MUP or MHC haplotypes shared with the female, her mother, or littermates (Table 1, test 7). Females preferred the related partner (p = 0.02; Figure 3N). There was no significant difference in this preference during different phases of the light cycle (p = 0.74), though it may be noted that preference was very consistent during the active dark phase (p = 0.003) and a little more variable when females nested together during the inactive light period (p = 0.09; Figure 3O). Thus, females also use other, as-yet-unidentified loci not closely linked to MUP or MHC to select relatives as preferred partners, though a small number preferred to nest with the unrelated female. Nonetheless, the consistency of overall preference for a female related at only r = 0.19 when no specific loci were selected to match to the subject suggests that this recognition involves sharing integrated across multiple additional unlinked alleles. This fits with general observations that overall similarity in complex mammalian individual odors co-varies continuously with the degree of genetic similarity between individuals, albeit with a high degree of variance [40–43]. As yet, it is not known whether

Table 2. Mixed-Effects Modeling of Nesting Partner Preferences

Dataset and Model	F Statistic	Probability
Figure 2B: Sister versus Unrelated (Familiar or Unfamiliar, n = 41)		
Relatedness	$F_{1,39.9} = 12.62$	*p = 0.001*
Familiarity[a]	$F_{1,30.1} = 0.51$	p = 0.48
Figure 3A: Full Self Match at MUP (n = 19)		
Match at MUP	$F_{1,16.3} = 12.61$	*p = 0.001*
Figure 3B: Full Self Match at MHC (n = 19)		
Match at MHC	$F_{1,17.8} = 0.26$	p = 0.69
Figure 3D: Haplotype Imprinting at MUP (n = 35)		
Maternal and littermate match at MUP	$F_{1,31.1} = 1.35$	p = 0.13
Figure 3E: Haplotype Imprinting at MHC (n = 33)		
Maternal and littermate match at MHC	$F_{1,27.5} = 5.27$	p = 0.99
Figure 3F: Full Maternal Match at MUP (n = 19)		
Match at MUP	$F_{1,16.0} = 0.76$	p = 0.20
Figure 3G: Full Maternal Match at MHC (n = 13)		
Match at MHC	$F_{1,9.9} = 0.55$	p = 0.76
Figure 3H and 3J: Partial Self/Paternal Match at MUP (n = 30)		
Match at MUP versus match at MUP and MHC[b]	$F_{1,27.2} = 1.21$	p = 0.14
Match at MUP	$F_{1,29.0} = 1.36$	p = 0.88
Figure 3I and 3J: Partial Self/Paternal Match at MHC (n = 28)		
Match at MHC versus match at MHC and MUP[b]	$F_{1,20.8} = 0.004$	p = 0.48
Match at MHC	$F_{1,28.0} = 0.16$	p = 0.66
Figure 3K and 3M: Partial Maternal Match at MUP (n = 26)		
Match at MUP versus match at MUP and MHC[b]	$F_{1,26.0} = 0.26$	p = 0.31
Match at MUP	$F_{1,22.9} = 0.36$	p = 0.25
Figure 3L and 3M: Partial Maternal Match at MHC (n = 28)		
Match at MHC versus match at MHC and MUP[b]	$F_{1,25.4} = 0.97$	p = 0.17
Match at MHC	$F_{1,15.2} = 0.57$	p = 0.77
Figure 3N: Partial Background (r = 0.19), no MUP or MHC (n = 16)		
Relatedness	$F_{1,\ 9.6} = 5.76$	*p = 0.02*

Results are presented for the fixed effect of greater time spent with the related or matching partner, with significant results shown in italics ($p < 0.05$). Other variables were included as random effects (subject ID, subject line, enclosure ID, matching at the non-focal marker, stimulus animal age, and weight difference) as relevant to specific models (see "Data Analysis" in the Supplemental Experimental Procedures).

[a]In the sister versus nonrelative model, the effect of familiarity on the bias in time spent with a sister versus nonrelative was tested by fitting an interaction term between relatedness and familiarity.

[b]In partial (single-haplotype) matching models, the effect of sharing at both markers was assessed first before pooling data to examine matching at the focal marker.

animals use an integrated similarity across all scent components to estimate relatedness or selectively assess scent components that correlate most strongly with sharing across the genome to provide the most reliable estimate of their degree of relatedness [41].

Investigation of Scent from Animals with Matching Kinship Markers

To examine whether females perceive a difference in urine scents of relatives due to similarity to own and known relative scents and whether this corresponds to matching at specific genetic markers, we compared initial investigation of urine from pairs of stimulus females. Tests were carried out before females met the scent donors themselves in our functional assay of nest partner preference. In agreement with studies in other species [42, 43], females spent less time investigating urine from an unfamiliar sister (r = 0.5) than that from an unrelated female during brief 10 min tests (p = 0.02; Figure 4B). The same discrimination was shown when the sister and unrelated female urine donors had been their familiar cagemates for at least 4 months prior to testing (p = 0.002; Figure 4A). Thus, investigation bias is not simply due to reduced "novelty" of scent from an unfamiliar sister, but reflects a persistent perception that scent from a close genetic relative requires less investigation due to its similarity to their own and/or familial odors imprinted during rearing. However, when relatedness was only r = 0.19 and the relative carried no MUP or MHC haplotypes that were familiar to the female, scent investigation was just as prolonged as that toward urine from an unrelated female (Figure 4C). Despite this, females still associated preferentially with the related female in nest partner tests (Figures 3N and 3O). We cannot distinguish whether this extended urine investigation provided information on kinship (which led to the association preference) or whether the cues used to recognize background relatedness were not detectable in urine.

When two donors were equally related to the female (r = 0.19 or 0.25), a match to the female's own MHC type (Figure 4E) or to one highly familiar maternal MUP or MHC haplotype (Figures 4F and 4G) failed to influence investigation of unfamiliar scent. By contrast, a match to the female's own MUP type encountered on a different genetic background increased (rather than reduced) the duration of investigation (p = 0.03; Figure 4D). Thus, recognition of MUP sharing was not simply due to greater familiarity of scent, distinct from the response to a sister's urine. The phenotype of involatile MUPs in urine was very similar between females sharing the same MUP genotype, even when their overall relatedness was only r = 0.19 (Figure S1). However, differences in other urinary volatile and peptide components at this level of relatedness were sufficient to stimulate as much investigation as an unrelated stimulus (see Figure 4C). Significant bias for more prolonged investigation of urine that matched the female's own MUP type on this different genetic background most likely reflects the processing time required to assess the similarity of an involatile MUP phenotype alongside other differences in a female's scent. It also confirms that females could detect sharing of MUP type through urine scent, providing a mechanism to select preferred nest partners based on shared MUP type.

DISCUSSION

We have shown that female house mice use genetic kinship markers to preferentially establish pre-reproductive nesting alliances with close kin, regardless of any prior familiarity. We have also shown that both MUP genotype and sharing at multiple

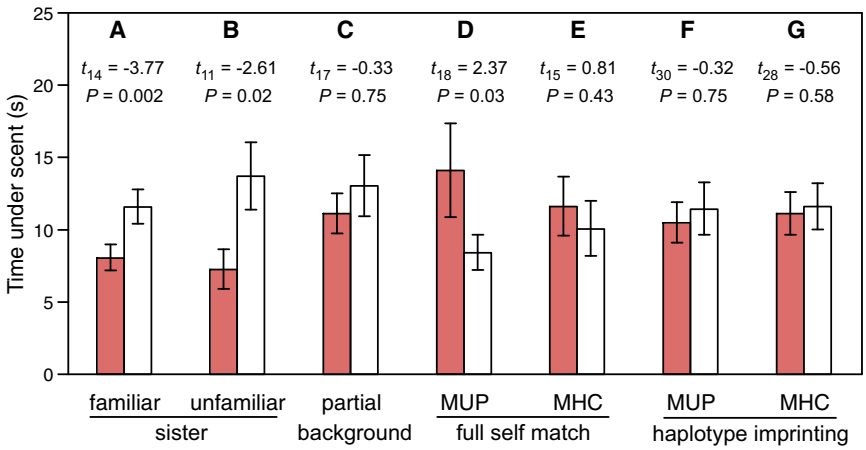

Figure 4. Discrimination of Urine Samples with Different Genetic Markers of Relatedness

Females were given a 10 min choice between 10 μl urine samples from a female with a specific marker of genetic relatedness (red filled bars) versus a control without (open bars), streaked on the ceiling on opposite sides of a divided arena. Time spent immediately under the urine sample (5.5 cm diameter circle) was recorded blind to urine identity (data are means ± SE). Urine choices shown were from a sister (r = 0.5) versus unrelated female when both donors were highly familiar (A) or both unfamiliar (B); a related (r = 0.19) versus unrelated donor where both carried novel MUP or MHC haplotypes that were not experienced by the subject during rearing (C); equally related females (both r = 0.19 or 0.25) that shared the same MUP (D) or MHC (E) type as the subject versus no MUP or MHC haplotype shared; or equally related females (both r = 0.19 or 0.25) that shared at least one MUP haplotype (F) or MHC haplotype (G) with the subject's mother versus a control that carried novel haplotypes not experienced by the subject during rearing. Matched-pair t tests compared time spent under the two stimuli in each test. Familiarity in sister tests had no effect on bias ($F_{1,25}$ = 0.26, p = 0.62). Urine donors in all other tests were unfamiliar. Example urinary MUP phenotypes for females sharing both, one or no MUP haplotypes are illustrated in Figure S1.

unidentified loci across the genome act as genetic kinship markers to establish these nesting partnerships. MUP genotype provides sufficient polymorphism to act as a kinship marker because of recent rapid expansion in the central region of the *Mup* gene cluster in commensal house mice, coincident with their separation from other *Mus* species [21]. In most other species examined to date, *Mup*-like genes show little or no polymorphism (in humans, there is only a single *Mup* pseudogene), although there has been completely independent expansion of these genes also in the Norway rat (*Rattus norvegicus*) [22]. Thus, MUP polymorphism is a species-specific signal comprising a set of specialized communication proteins that, in mice, are excreted in the urine of both sexes [27]. The individual scent signatures that MUPs encode also reflect close kinship through shared inheritance of tightly linked haplotypes. Importantly, these shared signatures are readily recognized against the heterogeneous genetic background of individual outbred animals (see also [23, 24]), a feature essential for genetic kinship markers. Although polymorphic MUP isoforms differ from each other by only a few amino acid changes [44], they are discriminated through vomeronasal sensory neurons using a combinatorial-coding strategy [25]. In addition, MUPs influence individual volatile odor signatures through binding and release of a wide range of urinary volatiles, with isoforms differing in specific binding affinities [45–48]. Further work will be needed to establish whether one or both of these mechanisms are involved in discriminating relatives that share the same MUP phenotype.

The rapid evolution of polymorphic MUP types in house mice most likely reflects strong selection pressure for reliable communication of both individual identity and close kinship in this social species. This will be particularly important in the context of cooperative breeding and communal nursing, when adult females make considerable investment in the offspring of others. That polymorphism in genetic markers could evolve specifically to promote nepotistic behavior (favoring of relatives) is controversial. The fitness advantage that is expected to accrue for common haplotypes could result in erosion of the variability required for recognition [49, 50]. Thus, it has been proposed that extrinsic processes must be necessary to maintain diversity in markers used for genetic kin recognition. For example, the primary role of MHC in immune function provides strong balancing selection to maintain its diversity, providing a polymorphic genetic marker that might then be used for kin recognition [50, 51]. However, mice did not use MHC sharing to select closely related nest partners, regardless of indisputable diversity at MHC. Instead, they used MUP sharing. Polymorphic MUP patterns in mice function only as a specialized communication signal. The use of MUP sharing to identify very closely related nesting partners, though, may be paralleled by a role for the same marker in inbreeding avoidance [3], although properly controlled tests like those presented here are still needed for confirmation. MUP polymorphism also provides an individual genetic signature that allows male mice to advertise their individual competitive ability through scent marks [23–25]. Frequency-dependent selection on MUP through roles in both inbreeding avoidance and individual recognition [52] could help to maintain variability among haplotypes necessary for the reliable recognition of closely related cooperative partners [53].

House mice use sharing at MUP in addition to shared background genes to discriminate preferred partners. In the absence of MUP sharing, those related across the genome are preferred to non-kin, but there is a strong preference for partners of equivalent relatedness that also share the female's MUP type. Inclusive fitness benefits gained from cooperating with relatives will depend on how closely related animals are, and thus the proportion of genes they share. A highly polymorphic locus like MUP is only likely to be fully shared between very close relatives, with increased likelihood of sharing if animals become more inbred and share a greater proportion of their genes. Thus, it is a reliable signal that relatedness across the genome is very high (most likely at least full sibs), even though close relatives will not all share the same type in outbred populations. Familial imprinting on MUP types during rearing could allow a greater range of relatives to be recognized than achieved just by self-reference.

However, animals are likely to encounter a wide range of relatedness in the nest due to frequent multiple paternity of litters in house mice [54, 55] and communal nesting even when closely related partners are not available [28, 29]. Imprinting on such cues would not provide the same reliable indicator of very close relatedness as a full match to themselves. When a full MUP match to themselves was not available, females preferred partners sharing at other loci not closely linked to MUP or MHC. Integration of sharing over multiple loci may allow animals to estimate their degree of genetic similarity [42]. However, the correlation between odor similarity and genetic similarity can be quite crude [40, 41] and could limit the sensitivity of this estimate. By contrast, full sharing at a single highly polymorphic gene cluster like MUP (or MHC) provides a simple reliable indicator that many genes are likely to be shared but cannot indicate different degrees of relatedness, as close relatives share the full range of none, one, or both haplotypes.

The absence of preference based on MHC sharing, whether through common inheritance or familial imprinting, will be surprising in view of the substantial literature showing that MHC type influences individual odors and social responses among laboratory mice [8, 14]. Indeed, the hypothesis that MHC odors provide a kinship marker stems largely from mouse studies [7]. An early influential study found that females rearing offspring communally in semi-natural enclosures had greater MHC sharing than a random model of partner choice among mice with a 50% wild-derived genetic background [18]. However, this was confounded with prior familiarity and genetic background that might also explain biases. Sisters previously reared together in cages could be removed from analyses, but there was no control of parentage and experience of those born in enclosures, background relatedness, or MUP sharing. By contrast, all of these factors were completely controlled with our approach. We could test directly (1) the separate effects of sharing MHC, MUP, and genetic background, (2) the effect of full-genotype or single-haplotype matching, and (3) reference to own genotype or familial imprinting. We found no evidence for any preference based on MHC matching, even in the most extreme choice of a full MHC match to themselves (which simultaneously includes maternal, paternal, and littermate matches, too) compared to no MHC haplotypes matched. Given that MHC and ESP regions exhibited strong linkage in our mice, this also implies that mice did not use *Esp* genes as a marker for kin recognition either. To date, there is no convincing evidence from mouse studies that MHC is used as a genetic kinship marker among genetically heterogeneous animals, or that MHC can provide a consistent kinship signature that is recognizable across different genetic backgrounds [24, 39, 56–59], in strong contrast to recognition of MUP type.

Evidence that other species use MHC as a genetic kinship marker is also surprisingly weak when correlations with genome-wide sharing have been controlled. Although MHC-homozygous (but not MHC-heterozygous) tadpoles of African clawed frogs associate preferentially with those of the same MHC type among familiar sibs [5, 10], they show the opposite preference for different MHC types among unfamiliar non-sibs [11]. As tadpoles from wild-caught parents show only very weak preference to associate with unfamiliar sibs over non-sibs [11], MHC preferences are unlikely to reflect genetic kin recognition. Similarly, there is some evidence that juvenile arctic char prefer the same homozygous MHC class IIb genotype among unfamiliar sibs, but no such discrimination was evident among non-sibs. Further, other unlinked genes were used to discriminate sibs from non-sibs when both shared the subject's MHC class IIb genotype [4, 9]. As sample sizes were extremely small (n = 5), further work is urgently needed to understand the influence of MHC sharing on social associations in arctic char and other species. The approach that we have demonstrated here could be applied to a wide range of vertebrates to test the use of MHC and other candidates as genetic kinship markers. While the idea that MHC could provide a vertebrate-wide genetic kinship marker is very attractive because of its potential generality, appropriately controlled evidence in support is sorely lacking. Instead, our study suggests that species-specific kinship markers evolve when there is strong advantage for reliable recognition of close kinship.

In conclusion, we have demonstrated that a species-specific polymorphic signal (MUP), but not MHC, is an important signal for discrimination of close kinship in the house mouse, on top of information provided by sharing at multiple loci across the genome. This calls for further investigation to establish the genetic markers that underlie kin recognition in other vertebrates. It remains to be discovered whether other species that breed cooperatively, in situations where related and unrelated animals mix, also evolve specific genetic kinship markers that allow reliable discrimination of those that are very closely related. In addition to identifying genetic markers and templates used for kin recognition in a cooperative context in the mouse, our study provides no support for the general assumption that MHC-associated scents provide a vertebrate-wide mechanism for kin recognition.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.08.045.

AUTHOR CONTRIBUTIONS

J.L.H., R.J.B., P.S., and S.P. designed the study and gained funding. J.L.H., A.M.H., and J.P.G. designed the behavioral experiments, which were performed by J.P.G., A.M.H., J.L.H., and A.J.D. S.P. and R.J.B. advised on genotyping and MUP phenotyping, respectively, which were carried out by A.J.D., J.P.G., A.M.H., and J.L.H. J.P.G. and J.L.H. carried out all statistical analyses. J.L.H. and J.P.G. drafted the manuscript, to which all authors contributed.

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Current Biology

Supplemental Information

The Genetic Basis of Kin Recognition in a Cooperatively Breeding Mammal

Jonathan P. Green, Andrew M. Holmes, Amanda J. Davidson, Steve Paterson, Paula Stockley, Robert J. Beynon, and Jane L. Hurst

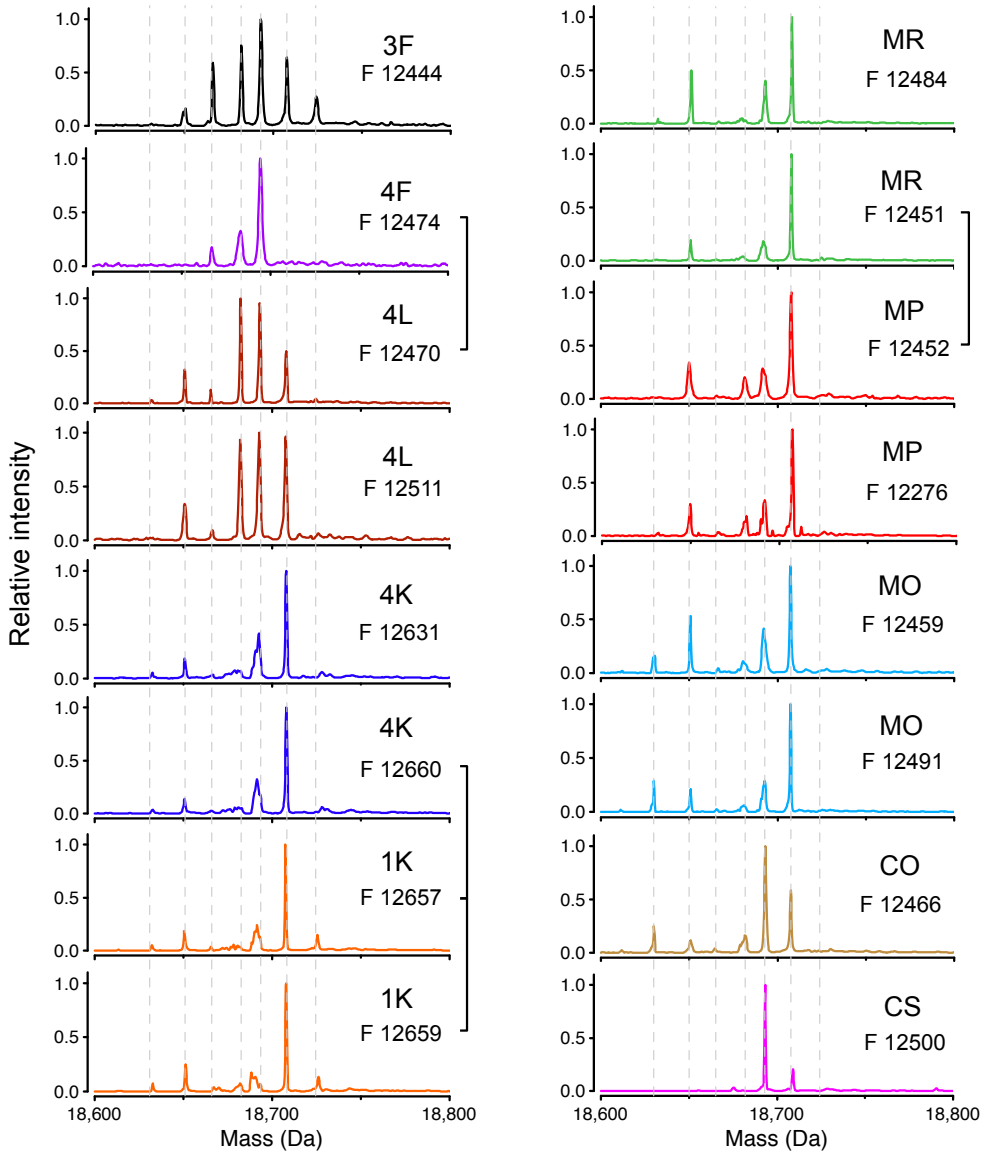

Figure S1. Typical MUP phenotypes of female house mice revealed by electrospray ionisation mass spectrometry of intact urine samples (related to Introduction and Figure 4). Intact mass spectra show the intensity of each mass peak relative to the highest peak in the spectrum for a typical selection of individual females in two unrelated lines (left, right). MUP genotypes were assessed independently using six microsatellite markers surrounding the MUP region, with haplotypes inherited from each parent assigned to an alphanumeric code (A-Z or 1-8). Within each line, females shown are 1.5x cousins (r = 0.19) except those linked by brackets which are full sisters (r = 0.5). Females that inherit the same MUP genotype express very similar MUP patterns regardless of degree of relatedness across the genome. The extent of phenotypic difference between haplotypes varied from several peaks not shared (e.g. 3F and 4F, left) to more subtle differences at a single peak (e.g. 4K and 1K, left; MR and MP, right) or in relative intensities of expression (e.g. MO and CO, right). Peaks of similar mass may contain more than one MUP isoform [S1], which could differ between haplotypes. Thus, shared haplotypes were defined through genetic pedigree analysis (shared inheritance), but ESI/MS confirms that different haplotypes usually encoded different phenotypes. Dashed lines show alignment of peaks at MUP masses 18630, 18651, 18666, 18682, 18693, 18708, 18725Da.

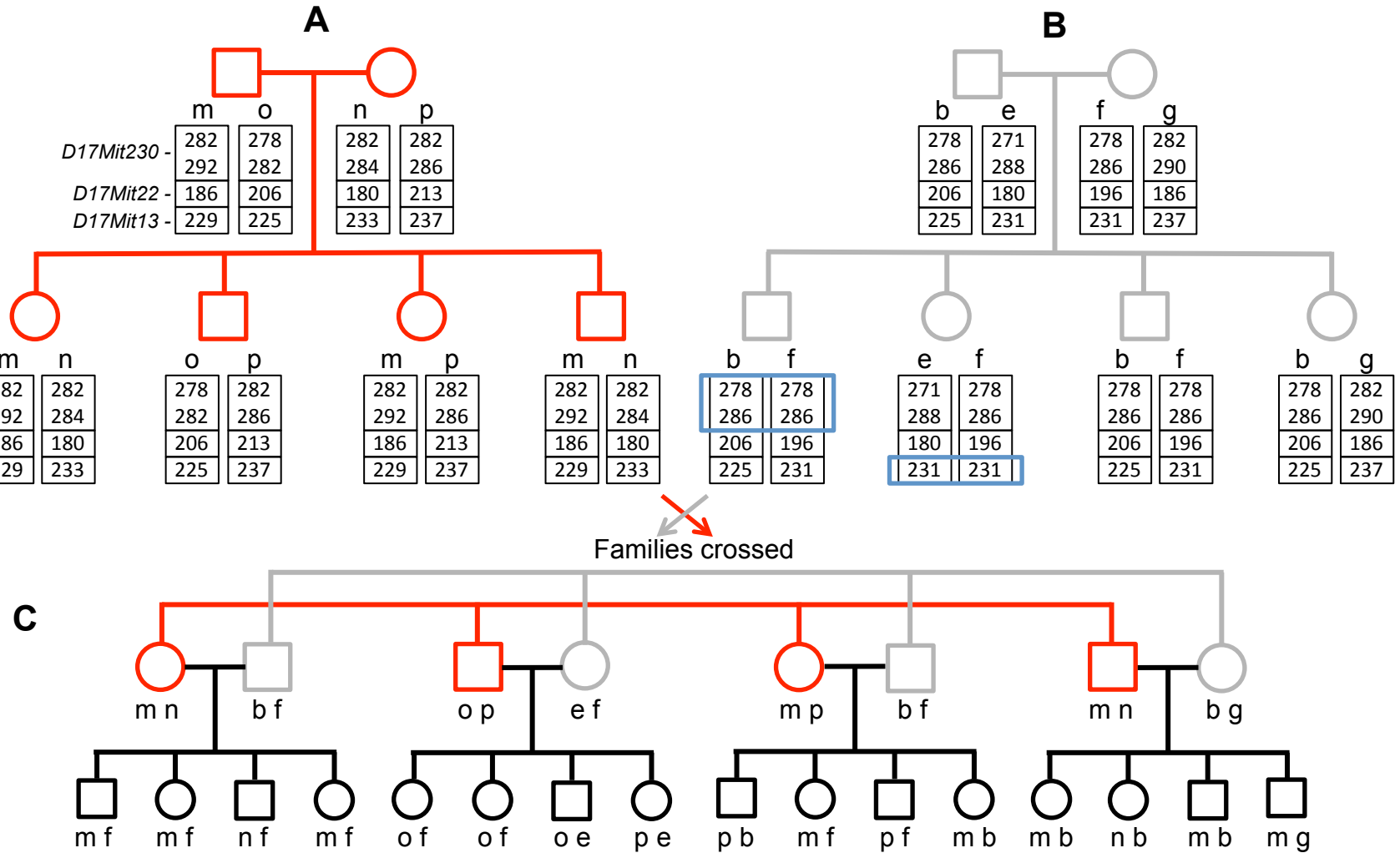

Figure S2. Generating family lines and haplotype tracking (related to Figure 2). Family lines were created by breeding two unrelated families of outbred sibs (**A**, **B**) which were then crossed (**C**) to provide multiple litters of unfamiliar double cousins (r = 0.25) as illustrated; or a set of sibs was crossed with an unrelated set of double cousins to provide multiple litters of unfamiliar animals related at r = 0.19 (not shown). Within a line, litters were equally related but each had different parents and family experience of MHC and MUP haplotypes. The example shows how MHC haplotypes (denoted by lower case letters) could be tracked from parents to offspring using a set of microsatellite markers across the MHC region (Figure S3) which were tightly linked. Identities of alleles are given as fragment sizes in base pairs. Note that *D17Mit230* provided a double marker. Occasionally, a parent had the same alleles at the same microsatellite in two different haplotypes (blue boxes), where a recombination event would not be detected, but recombination events were very rare (0.2% for MHC haplotypes, 0.7% for MUP haplotypes when cross-over events would be seen). MUP haplotypes were tracked similarly.

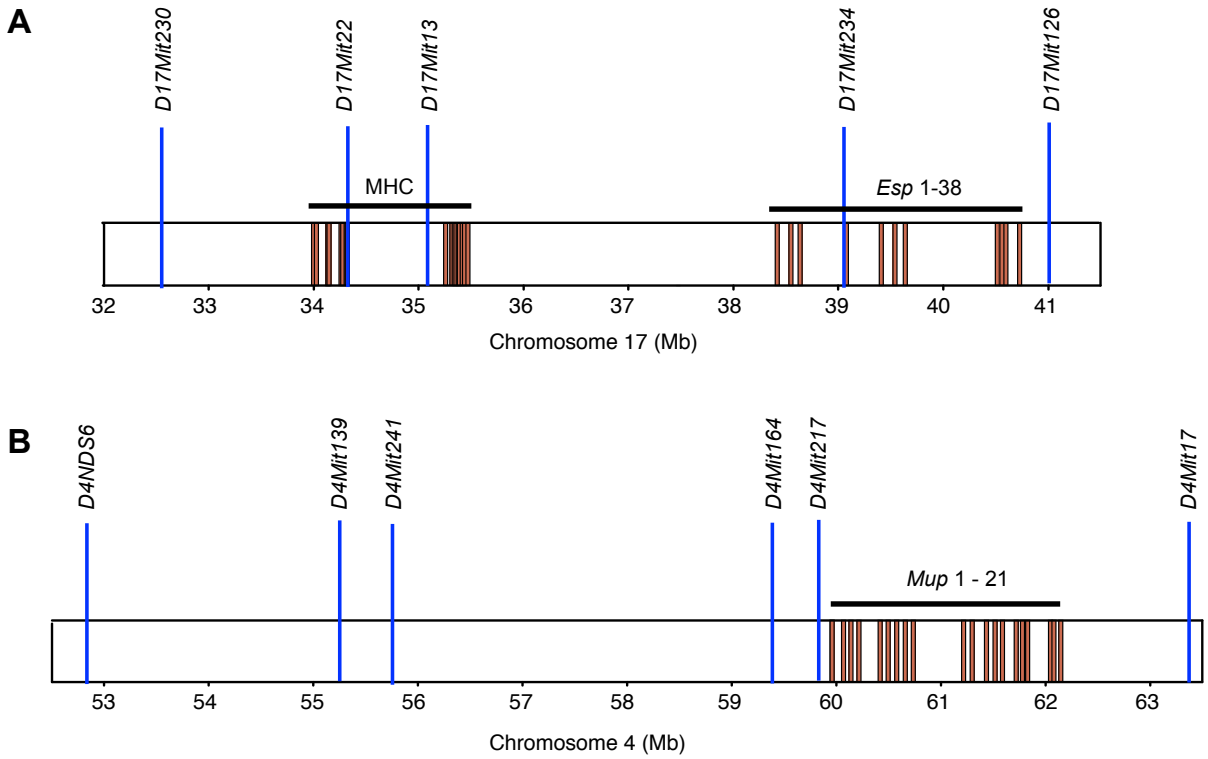

Figure S3. Location of microsatellite markers used to track MHC, ESP and MUP haplotypes (related to Figures 2, S2 and Table S1). Blue lines indicate location of microsatellite markers (Table S1), brown lines indicate location of MHC (= *H-2*), *Esp* and *Mup* genes. Three microsatellite markers (one double) were located across the region encoding classical MHC class 1 and class 2 genes and two were in the *Esp* gene region on mouse chromosome 17 (**A**). A previous study using an additional marker at 37.48Mb (*D17Mit24*) revealed that an additional marker to the right of *D17Mit13* was not necessary to track haplotypes between parents and offspring as recombination events over this distance were very rare [S2]. Six microsatellite markers were located on chromosome 4 to track MUP haplotypes (**B**). Only recombination events between *D4Mit217* and *D4Mit17* affected MUP haplotype, but additional markers were necessary to track the different haplotypes carried (no microsatellite markers are located within the *Mup* gene region itself). ESI/MS confirmed that haplotypes defined by different patterns of microsatellites close to *Mup* genes had detectable differences in phenotype (Figure S1).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Subject and stimulus animals

Test animals were 236 captive-bred female *Mus musculus domesticus* (F2-F5) derived from ancestors captured from five populations in the northwest of England, UK. Ancestors were captured within 20 miles, except for two individuals that were captured 80 miles away. During rearing, females were housed with mothers and litter-mates. From weaning (aged 24-28 days), females were housed in 45 x 28 x 13cm cages (MB1, North Kent Plastics, UK) in single-sex small family groups (2-4 animals). Throughout, females were tested with unfamiliar stimulus animals housed in different caged groups except when tested with a familiar sister versus familiar unrelated female (see details below). Mice were maintained on Corn Cob Absorb 10/14 substrate with paper wool nest material and *ad libitum* access to water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, St Louis, MO, USA). Cardboard tubes and red plastic mouse houses (Tecniplast UK Ltd) were provided for home cage enrichment. Animals were housed on a reversed 12: 12 h light cycle with lights off at 08:00. Each female was injected with a radio frequency identification (RFID) tag beneath the skin at the nape of the neck for individual identification and to automatically track location in nest partner preference tests.

Sisters versus unrelated females

We first tested whether outbred wild-stock house mice showed a nest partner preference for full sisters ($r = 0.5$) over unrelated females ($r < 0.04$), and whether the same preference was shown whether females were previously familiar or not with the stimulus animals. To create groups of familiar females, two unrelated pairs of littermate sisters (obtained from 16 unrelated litters) were housed together from weaning, reflecting the mixing of animals in the wild once they become independent. Subjects therefore had cohabited with a familiar sister stimulus from conception and with a familiar but unrelated stimulus from 24-28 days of age. Unfamiliar sisters were from a previous litter, approximately one month older than the subject, while unfamiliar unrelated females were age-matched to the unfamiliar sister stimulus. This allowed us to test (i) whether females preferred to nest with an unfamiliar close relative over a matched unrelated female, implying the use of genetic markers, and (ii) compare the strength of this preference to that shown between familiar females where animals could use individual cues learned during rearing as well as genetic markers to recognize relatives.

Kinship marker experimental design

We focused on MHC and MUPs because they are known to influence mouse scents and have sufficient polymorphism to provide kinship markers. To test whether either or both are used to recognise unfamiliar kin as preferred nest partners, we needed to compare preference between equivalent unfamiliar animals that differed in their match at one of these polymorphic markers while controlling for matching at the other. We also needed to match relatedness across the genome to control for any unknown genetic markers of relatedness that animals might be using, and independently test whether animals use such background relatedness when sharing at MHC and MUP could not be used. It is not feasible to fully sequence complex gene clusters such as MHC or MUP across large numbers of genetically heterogeneous animals. Instead, many previous studies have used inbred laboratory strains to generate animals carrying a very limited set of laboratory-derived MHC haplotypes that could easily be typed using a single marker or small number of markers (e.g. [S3, S4, S5, S6]). Here we use a different approach: by tracking the sharing of MHC and MUP haplotypes through recent common descent, we could reliably assess the normal sharing that arises from close kinship at these specific regions of the genome even among outbred animals that carried natural polymorphic haplotypes recently derived from the wild. This allowed us to model the situation in most natural populations where (i) individuals are generally heterozygous at the highly polymorphic loci required for kin recognition, (ii) kinship markers must be recognized in other unfamiliar individuals on randomly assorting genetic backgrounds, (iii) parents and offspring within families individually carry different combinations of inherited haplotypes, and (iv) animals experience different sets of haplotypes in each family group during rearing. These are key differences to laboratory studies using inbred strains. Our breeding design generated family lines of unfamiliar outbred female house mice that had the same level of overall relatedness but came from different outbred parents; individuals varied both in their sharing at candidate genetic markers and in the specific haplotypes they experienced during rearing (Figure S2). By comparing responses to equivalent unfamiliar females from the same family line, we used known pedigree to fully control for the natural correlation between sharing at candidate loci and average sharing at other potential markers across the genome, and had very strong confidence that any haplotypes shared were identical by descent (see Genotyping methods below).

As animals could recognize kin using a self-referent matching mechanism (comparing the phenotypes of other animals to themselves) and/or familial imprinting (matching

phenotypes to familiar relatives they were reared with), we needed to manipulate whether matching at a candidate marker was to self or to familiar family members. Previous studies that have attempted to address whether familial MHC markers learned during rearing subsequently influence the selection of mates in adulthood have used cross-fostering designs, whereby individuals or litters of animals were transferred between mothers with different MHC genotypes shortly after birth [S4, S5]. A major limitation of this approach is that it confounds experience of recognition cues acquired after birth with those acquired *in utero* [S7], which is problematic where formation of a recognition template depends upon combined exposure to pre- and post-natal cues (e.g. [S8]). It also conflicts self-referent cues with those from the rearing environment, both of which could contribute to kin recognition through phenotype matching. The approach used here avoids these issues by allowing animals to experience their natural family cues during development, thereby permitting normal acquisition of recognition cues and the development of recognition templates. We then tested specific candidate markers by careful selection of matching versus non-matching stimulus animals for each individual subject according to the specific haplotypes that were carried by the subject and those they had previously experienced during rearing.

Candidate markers and family lines

Parents and their offspring were genotyped at three candidate loci: the major histocompatibility complex (known as *H-2* in mice, but referred to here as MHC), the major urinary protein (MUP) cluster and the exocrine-gland secreting peptide (ESP) cluster, which is another cluster of *Esp* genes that encode pheromones known to be involved in mouse olfactory signaling, although phenotypic variation in ESP expression has only been examined in laboratory strains so far [S9]. *Mup* genes are on mouse chromosome 4 and inherited independently from MHC and ESP on chromosome 17. Among our wild-stock animals, MHC and ESP markers were very tightly correlated (see *Genotyping Methods and Linkage* below). This prevented us from testing their independent effects through normal breeding. Hereafter, these tightly linked gene clusters are referred to simply as MHC, but any effects due to MHC could potentially be explained by differences in MHC and/or ESP-type (or any other very closely linked genes that influenced phenotype). Conversely, an absence of MHC effect implies that ESP did not provide a marker used for kin recognition either. Other genes closely linked to the MUP cluster on chromosome 4 are not currently known to influence olfactory signals, but it is possible that other closely linked genes could contribute to effects due to shared inheritance of MUP haplotypes.

To create each of five different outbred lines, we mated a set of full siblings with another unrelated set of full siblings to generate double cousins (r = 0.25, see example in Figure S2) or mated a set of full siblings with an unrelated set of double cousins to generate 1.5x cousins (r = 0.19). To reliably follow individual haplotypes by descent within each family using microsatellite markers, each parental haplotype needed to be identifiable through a unique set of microsatellite alleles. Thus, within each family line the founding parents were unrelated to ensure that they carried a range of MHC and MUP haplotypes and we used sufficient microsatellite markers to reliably distinguish between haplotypes inherited within each family group. Importantly, the same set of microsatellite alleles in two different individuals can only be assumed to represent identical haplotypes if they are known to derive recently from the same heterozygous ancestor and followed through each generation to check for uncommon recombination events. By tracking each haplotype through shared descent, we could be certain that shared haplotypes were identical (Figure S2); we did not use pairings where animals shared the same set of microsatellite alleles that did not originate from a recent common ancestor.

Assessment of kin recognition markers: tests

We first tested for the use of MHC and/or MUP markers in nest partner preference by giving females a choice of an unfamiliar stimulus that fully matched the subject's MHC or MUP type (i.e. both haplotypes at the focal marker shared) versus a stimulus of equal relatedness from the same family line (either r = 0.19 or r = 0.25) that did not match at the focal marker (neither haplotype shared); matching at the other marker was identical for both stimulus females (see example in Table 1: test 1; Figure 2B). In this initial general test, recognition of the focal marker (MHC or MUP) could be due to a number of different mechanisms or a combination of these, including self-referent matching and/or imprinting on familial haplotypes experienced during rearing.

A series of subsequent tests investigated whether the mechanism (a) was self-referent and/or due to familial imprinting, and (b) required a full match of both haplotypes (i.e. genetically identical) at a particular marker or whether a match of single haplotypes (partial match) at one or both markers was sufficient. Females were given a choice between two equally related stimulus females that differed in matching at MHC and/or MUP to either the subject herself, or to the maternal genotype that the subject experienced during rearing (Table 1: tests 2-6). As subjects inherit only one maternal haplotype at each marker, recognition of a single haplotype maternal-match (inherited by all maternal littermates) was tested using

matching of the maternal haplotype that the subject herself did not inherit to eliminate the use of self-referent matching in these tests (Table 1: tests 2 and 6). We focused on a maternal match as this is the kin template that all mammals reliably experience *in utero* and during lactation, while littermates carrying the same maternal haplotypes may also reinforce this learned template. If single haplotype sharing with any animals familiar during rearing is used (including self and littermates), the matching stimulus animal should be recognised in all partial maternal-match and partial self-match tests (Table 1: tests 2, 4, 5 and 6). In house mice, post-natal learned imprinting on paternal haplotypes is unlikely to have evolved as a reliable mechanism of kin recognition because multiple (extra-territorial) mating by females occurs frequently [S10] and adult males (paternal or unrelated) are variably present in the nest (adult males were not present during rearing in the current study). However, there is some evidence in humans (not in the context of kin recognition) that women discriminate between odours from men of different ethnicity to themselves according to the number of MHC alleles shared, based on the woman's own paternal inheritance of matching alleles but not on maternal inheritance or on post-natal learning of paternal or maternal odour [S11]. Thus, a partial self-match based on the subject's paternally inherited haplotype (Table 1: tests 4 and 5) might be recognized because of the familiar match to self and/or littermates, or because paternally-inherited alleles play a special role in recognition regardless of the sire's presence during rearing. Comparison between a match to a subject's paternally or maternally inherited haplotype would help to distinguish between these mechanisms. In all tests, both haplotypes of the nonmatching stimulus animal differed from the subject and from the subject's mother at the focal marker, while average similarity at non-focal markers was controlled. As an overall test of familial imprinting (familiarity with any haplotypes experienced in the nest but not carried by the subject herself), we pooled responses in all trials above where the matching stimulus carried at least one haplotype that matched the subject's mother and littermates (thus was strongly familiar during rearing), while the non-matching stimulus carried two novel haplotypes that had not been experienced at all by the subject during rearing.

Finally, to test recognition using loci other than MUP or MHC or genes closely linked to these clusters (referred to here as genetic background), neither stimulus animal carried MHC or MUP haplotypes that were shared with the subject, or were previously familiar to the subject during rearing. The matching stimulus animal was the subject's 1.5x cousin ($r = 0.19$) while the nonmatching stimulus animal was age-matched but bred through an unrelated

line (Table 1: test 7). Recognition using sharing of unknown background genes could involve self-referent and/or familial matching.

Behavioural bioassays

Kin recognition was tested in a functional context by determining the preference of subjects for nesting in close proximity to a matching versus a control non-matching stimulus female. Animals were aged 11.5 ± 0.25 months (mean ± SE) and in good health at the time of testing, with pairs of stimulus females age-matched as far as possible (0.01 ± 0.12 months age difference). Subject animals were used only once in each type of test and stimulus animals were used only once in the same role in each test. Sample sizes were determined by the availability of animals that met the strict matching and control criteria for each test. Females were previously familiar only in the test of recognition between familiar sisters.

Nest partner preference tests

Subject females were placed in a central cage (48 x 15 x 13 cm) connected to two side cages (45 x 28 x 13 cm) by clear acrylic tunnels so that subjects could move freely between the three cages (Figure 1A). Stimulus females were placed behind barriers made of mesh and clear acrylic (45 x 13 cm) that divided each side cage laterally and permitted visual, olfactory and limited physical contact between the subject and stimulus females. This allowed partner preferences of the subject female to be assessed independently of the preferences of the stimulus females. Matching and non-matching stimulus females were assigned randomly to left and right side cages for each replicate. If neither partner was attractive, subjects could choose to nest alone in the centre cage. Food and water were provided *ad libitum* in the centre cage and in each half of both side cages, so that each female had her own food and water supply and the subject female had access to food and water in each cage. A small amount of nest material was provided in each section of each cage. Aluzinc covers placed over the side cages, in addition to the food and water hoppers in each mesh cage top, provided females with shade during the light phase. A custom-built automated RFID data-logging system was used to record the location of subjects for the duration of each trial (for details see [S12]). An automated RFID reader housed in a clear Perspex box (110mm x 126 mm x 115 mm; Francis Scientific Instruments, Cambridge, United Kingdom) was placed around each connecting tunnel. Each RFID reader was paired with an infrared beam and associated detector, which allowed us to assign the direction of movement and hence the location of a subject in each of the three cages. Each time a subject passed through a detector,

the unique RFID code, time, date, and direction of travel were logged to a central computer. The sequence of movements for each subject was subsequently analysed blind to the location and identity of each stimulus animal (software written by JLH) to give the total time spent within each cage as a proportion of the total recording time, and when divided into the light and dark phases of the light cycle. Replicate cage systems were each housed separately in one of sixteen indoor enclosures (1.2m x 1.2m) to isolate each trio of females. Initial tests assessing preference for nesting with sisters over unrelated females were run for 72 h. In subsequent tests, this was reduced to 36 h because we found that this was sufficient time for stable preferences to be established. All trials started within the last 5 h of the dark phase. In some trials (26%), a centre cage of size 45 x 28 x 13cm was used, but this had no impact on the proportion of time spent in the side cages.

Urine discrimination tests

To examine evidence that females discriminated differences between the urine scents of stimulus females according to their relatedness or match at a specific marker, we carried out brief (10min) tests of discrimination between urine samples from pairs of unfamiliar stimulus females immediately before each test of nest partner preference. Previous studies report that a correlation between genetic similarity and odour similarity results in proportionately shorter investigation when animals first encounter scent from an unfamiliar relative (e.g. [S13, S14, S15]). Accordingly, this predicts that females will spend less time investigating unfamiliar scent from a matching donor compared to a non-matching donor because of its reduced novelty [S16]. However, attraction to associate with close relatives from a functional perspective leads to the opposite prediction, that females will spend more time near the matching stimulus scent. Either of these responses would confirm that females are able to discriminate between matching and non-matching females based on differences in urine scent.

Test procedures were modified from Ramm *et al.* [S17]. Urine, collected by allowing stimulus animals to urinate freely in clean transparent cages with a grill over the base, was stored frozen at -20^{o}C until use. Tests were performed in clean 45 x 28 x 13cm arenas (MB1 cage bases fitted with clear perforated Perspex lids). A clear acrylic barrier (28 x 13cm) divided the arena in half, with a circular hole (5 cm diameter) in the centre of the barrier to allow movement from one side of the arena to the other. Subjects were habituated to the test arena with a clean lid prior to testing. Urine (10 µl) from the two stimulus donors was streaked in the same orientation onto separate pieces of filter paper (5.5 cm diameter glass

microfiber filter cut in half), fastened with Sellotape on the underside of the test lid in opposite sides of the arena, 25 cm apart. The position of matching and non-matching stimulus urine was randomised but balanced. Urine was given two minutes to dry before the subject was presented with the test lid. Trials lasted 10 minutes and female behaviour towards the two scents was recorded remotely to DVD. Time spent directly under each scent stimulus (within a 5.5cm diameter circle) was transcribed manually from recordings using an event recorder program by an observer blind to the position of each scent.

Data analysis

Nest partner preference tests

Analyses were performed in R (v.3.1.0) (R Core Team 2014) or SPSS (IBM version 21). Preference for the related or matching nesting partner was assessed as greater time spent with the matching versus non-matching partner (expressed as a proportion of total test time in the respective side cage). We ran linear mixed-effects models (lmer function in lmerTest package) on the difference in time spent in the two side cages as the response variable. Relatedness or matching (yes or no) was fitted as the predictor in the full model. In the sister vs. nonrelative model, the effect of familiarity on the bias in time spent with sisters versus nonrelatives was assessed by including the interaction between relatedness and familiarity in the full model; the effect of relatedness was then assessed following removal of this interaction term from the model. Age and weight differences, and identity of the test enclosure were fitted as random factors in all models, with subject line as a random factor in all but the sister vs. nonrelative model. In the pooled familial haplotype imprinting models (based on a haplotype match with the subject's mother and littermates versus novel haplotypes), matching at the non-focal marker was included as a random factor to control for the minority of trials where there was also matching at the other marker. In pooled partial matching models, we first confirmed there was no difference between matching at one versus both markers. We then test the overall effect of matching at the focal marker, with match at the other marker included as a random factor. In analyses that pooled data over different tests, the occasional repeated use of subjects was controlled by including subject identity as an additional random factor. Where this was not possible due to small numbers of subjects used repeatedly, analyses were repeated omitting trials that used the same subject, which confirmed in each case that repeated use of subjects did not affect the results. *F* statistics and associated *P* values were obtained for effects of relatedness / matching in each model using the anova function. Results of all models are presented in Table 2. *P*-values for the effect of

relatedness / matching in all models are one-tailed to address our directional question of whether subjects spend more time with the related / matching partner. Note that the directional (one-tailed) test of greater time with the matching nesting partner is conservative for concluding that there was no discrimination between nesting partners when discrimination might be weak. In addition, simpler non-parametric Wilcoxon matched-pair tests confirmed very similar *P* values for the effect of relatedness / matching for all of the models tested (not shown), making no assumptions about the probability distributions of the responses assessed.

Urine discrimination tests

To assess initial discrimination between urine scents from the two stimulus females, paired *t*-tests compared the time spent directly under each scent, log transformed (s+1) to meet assumptions of normality (Shapiro-Wilks tests, $P > 0.05$). Tests of scent discrimination were two-tailed because we did not have a clear directional prediction for female responses to the two scents (see test description above). Some trials were eliminated *a priori* when mice failed to visit one or both stimuli (16 trials), showed stereotypical behaviour (8 trials), chewed the filter paper (2 trials) or a urine sample could not be obtained (1 trial). Across all trials, no bias was observed for a particular side of the test arena (1-sample t test, $t_{171} = 1.09$, $P = 0.28$).

Genotyping methods and linkage

MUP, MHC and ESP haplotypes were established by genotyping parents and offspring using microsatellite markers selected from the Mouse Genome Informatics site (MGI 5.1.3). These had previously shown sufficient polymorphism to reliably discriminate different haplotypes through descent within known pedigrees of wild UK *M. musculus domesticus* [S2]. One double and two standard microsatellites (equivalent to four markers) were selected to span the MHC region on chromosome 17; two additional markers were located within the ESP region in the neighbouring region of chromosome 17 (Figure S2A). As there are no microsatellites within the MUP region itself on chromosome 4, six markers were selected on either side of the MUP region to track MUP haplotypes (Figure S2B). DNA was extracted from a 5 mm ear punch using a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, West Sussex, UK) following the manufacturer's instructions. The forward primer for each marker was 50-fluorescently labelled with 6-FAM, NED, PET, or VIC. The loci were organized into three multiplex loading groups, containing mixed loci from the three regions. PCR amplification reactions were performed in a 10 μl volume of 20 ng DNA, 0.5 μM primer and 5 μl of BioMix Red reaction mix (Biolin, London, UK). The Touchdown PCR protocol steps

Microsatellite markers used for MHC, ESP and MUP genotyping

MHC Marker	Location[a]	Forward Primer	Reverse Primer	Size	Repeat	Label
D17Mit230	Chr17 32.56 Mb	5'-GCCTCAGCAAGAC CCTAAAC-3'	5'-CTCCTCCTTTTCC CTCTCC-3'	285	GT	PET
D17Mit22	Chr17 34.33 Mb	5'-GCATTAGATAGAG AGTAGATGGGTTG-3'	5'-TGGATGGCGAGA ATGAGAC-3'	216	GT	VIC
D17Mit13	Chr17 35.08 Mb	5'-TGCAGGCAAGATC CAAGAAG-3'	5'-GAAAGAGGGTGT CGATGCTC-3'	239	GT	FAM
ESP Marker						
D17Mit234	Chr17 39.05 Mb	5'-GCAAAGACAAA AATTGAAATGTG-3'	5'-CTGCTTAGCACAC ATGCTTTG-3'	115	CA	NED
D17Mit126	Chr17 41.00 Mb	5'-TATGTGGCATCTCT TTATTCATGA-3'	5'-CCAAGGATTGTC TGCCTTA-3'	131	CA	VIC
MUP Marker						
D4NDS6	Chr4 52.84 Mb	5'-CGGGGAAGGTTGT TTGTTTG-3'	5'-GGCCAGCAATGT AGAAAGG-3'	240	GT	VIC
D4Mit139	Chr4 55.25 Mb	5'-TCAAACTGGGAAG AGCCAAG-3'	5'-GCCGTAGAAGAG AAGTAATTTTTCC-3'	149	GT	PET
D4Mit241	Chr4 55.76 Mb	5'-TTTCCAGTGTTGTC CAGAGC-3'	5'-AGGCAAATCACT AGGTGCTG-3'	219	CA	FAM
D4Mit164	Chr4 59.40 Mb	5'-AACACATATATAC CAAGGCAGCAC-3'	5'-ATTTCCACCCTGT CCACTCC-3'	142	CA	NED
D4Mit217	Chr4 59.83 Mb	5'-ACTCAATTAGGTT GTTCAGATAGCC-3'	5'-GGCACTTGCTGCC ACATC-3'	246	GT	NED
D4Mit17	Chr4 63.37 Mb	5'-GCCAACCTCTGTG CTTCC-3'	5'-CCTCTGACATCCA CACACATC-3'	138	GT	FAM

[a] Locations are given for mouse genome reference assembly GRCm38

were: an initial denaturation for 5 min at 94 °C; 35 cycles of 30 s at 92 °C, 30 s at 60 °C (decreasing 0.1 °C per cycle to 56 °C), 30 s at 72 °C and a final extension of 10 s at 72 °C, followed by a 10 °C hold. The PCR reactions were then diluted to 25- to 50-fold (depending on primer set) and multiplexed in formamide with GeneScan LIZ500 size standard (Applied Biosystems). Microsatellite allele sizes were determined with an ABI PRISM 3100 DNA analyzer and GeneMapper v3.0 software (Applied Biosystems). The number of haplotypes identified in each breeding line is given in the table below.

Number of MUP, MHC and ESP haplotypes in each of five genotyped family lines

Line	MUP[a]	MHC[b]	ESP[b]
A	8	4	3
D	9	8	5
E	10	9	6
DE	8	7	5
ED	10	6	6

[a] Phenotyping by ESI/MS confirmed that different MUP haplotypes within a family determined by microsatellite sequencing corresponded to phenotypic differences.
[b] MHC and ESP haplotype numbers were determined by microsatellite sequencing.

MHC and MUP haplotypes are inherited intact as very tightly linked clusters of genes on different chromosomes. Figure S2 shows a typical example of how specific haplotypes could be tracked from parents to offspring using these markers. Recombination events could be detected as long as alleles at a particular marker differed between the two haplotypes carried by a particular parent. In the example shown, recombination events could have been detected in almost all cases, except when a parent carried a combination of b and f haplotypes (same alleles at *D17Mit30*) or e and f haplotypes (same allele at *D17Mit13*). However, recombination events were rare. Recombination rates were calculated based on the inheritance of haplotypes when no recombination event could have been concealed because of shared parental alleles. In the MHC region (not including ESP), only one recombination event was observed out of 405 haplotypes tracked from parent to offspring where a recombination event between markers could not be concealed, giving a recombination rate of 0.2%. Around the MUP region, recombination between markers *D4Mit217* and *D4Mit17* would influence the MUP cluster itself. We observed 5 recombination events in 676 inherited haplotypes where a recombination event could not be concealed, giving a recombination rate

of 0.7% within the MUP region. As recombination was so rare within both gene clusters, it is very unlikely that we missed recombination events even when this could have been concealed.

As MHC and ESP regions were close together on chromosome 17, we tested linkage using linkage disequilibrium tests (default settings: batches = 100, iterations/batch = 5000) in Genepop v. 4.0 [S18, S19]. MHC and ESP were very tightly linked in all five breeding lines (G-tests, $P < 0.0001$ in each case).

Electrospray ionization mass spectrometry (ESI/MS)

It is not yet possible to define MHC odour phenotypes in mice, although it is well established that MHC in laboratory mice influences volatile urinary odours [S20] and urinary peptides [S21, S22], both of which can be discriminated by mice when encountered on a constant genetic background. Phenotypic polymorphism in MUPs corresponding to MUP genotype can be detected as different patterns of MUP isoforms, defined by small differences in the masses and/or charges of MUPs expressed by genes in the central region of the MUP cluster [S23, S24]. Previous studies of the effects of MUP genotype on MUP phenotype have focused largely on males. To confirm that similarity or difference in MUP genotype between animals (defined by inheritance of the same or different microsatellite markers) corresponded to phenotypic similarity or difference in females, we examined the profile of MUPs expressed in the urine of subjects and stimulus animals using electrospray ionization mass spectrometry. This separates MUPs in each urine sample that differ in mass by 1-2Da or more, though does not resolve MUPs that share very similar masses (some MUP isoforms are known to share very similar masses [S23]). Urine samples were diluted 1:100 in 0.1% (v/v) formic acid. All analyses were performed on a Nano Acquity Ultra performance liquid chromatography system (UPLC; Waters, Wilmslow, UK) coupled to a QToF micro mass spectrometer (Waters), fitted with an ESI source. Samples (6 µL) were desalted and concentrated on a C4 reverse phase trap (LC packings). MUPs were eluted at a flow rate of 40µl/min using repeated 5 - 95% acetonitrile gradients. Data were gathered between 800 and 1600 Th and were processed and transformed to a true mass scale using MaxENT 1 maximum entropy software (Waters Micromass, Massachusetts, USA). All data sets were processed at 0.25 Da/channel over a mass range of 18400 - 19000 Da, and a peak width of 0.75 Da was used to construct the damage model. The calibrant was 2pmol/µL solution of horse heart myoglobin (Sigma, Munich, Germany), 2 mM DTT in 0.1% (v/v) formic acid. All water used was HPLC grade (VWR International, UK). Examples of these analyses are

shown in Figure S1. This confirmed that animals that shared both MUP haplotypes had very similar MUP phenotypes (MUP peaks matched) while those with non-matching MUP genotypes (different microsatellite sequences) generally had different phenotypes (not all MUP peaks matched).

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