Chem. Senses 36: 125–135, 2011
doi:10.1093/chemse/bjq103
Advance Access publication October 26, 2010

Urine-Derived Key Volatiles May Signal Genetic Relatedness in Male Rats

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Accepted August 26, 2010

Abstract

Olfactory cues play a vital role in kin recognition and mate choice of the rat. Here, using 2 inbred strains of rats, Brown Norway (BN) and Lewis, as models to simulate kinship via genetic distance, we examined whether urine-derived volatiles are genetically determined, and, if so, how they code for olfactory information and the degree of genetic relatedness in mate choice. Binary choice tests showed that BN females preferred the urine odor of Lewis males over that of BN males, suggesting that they avoided males genetically similar to themselves and were able to assess this olfactorily. Gas chromatography–mass spectrometry analysis revealed that the composition of urine-derived volatiles was more similar within strains than between strains and suggests that odortypes may reflect genetic relatedness. Our data further show that BN males had lower ratios of 2-heptanone and 4-heptanone and higher ratios of dimethyl sulfone and 4-ethyl phenol than Lewis males. When we supplemented BN and Lewis male urine to make each similar, the preferences of BN females were reversed. We conclude that some urine-derived volatiles covary in relative abundance with degree of genetic relatedness, and this relationship may play a key role in chemical signaling and genetic identity in this species.

Key words: chemosignal, genetic identity, mate choice, rat, urine

Introduction

Kin recognition and discrimination allow animals to distinguish between kin and nonkin using conspecific cues (Hepper 1991b). Several cue-based mechanisms are responsible such as social familiarity via prior association during early development (e.g., siblings and parents) and genetically determined-phenotype matching (Hepper 1991a; Mateo 2003). In rodents, body odor is an important cue mediating sociosexual behavior (Brown 1985; Hepper 1991b). There is considerable evidence to support phenotype matching in a variety of rodent species, such as golden hamsters (*Mesocricetus auratus*), Belding's ground squirrels (*Spermophilus beldingi*), and beavers (*Castor canadensis*) (Holmes 1986; Sun and Müller-Schwarze 1997, 1998; Mateo and Johnston 2000; Mateo 2002). Animals with multiple paternity or maternity may best assess their relatedness to unfamiliar conspecifics by comparing their own odortypes released from specialized scent glands with those of unidentified individuals (so-called phenotype matching) (Mateo and Johnston 2000).

Kin recognition and discrimination are important for mate choice and allow for the optimization of inbreeding and outbreeding (Hepper 1991a). Unrelated individuals are likely to possess different genotypes, and animals avoid breeding with close relatives to ensure offspring heterozygosity (Pusey and Wolf 1996). It has been well documented in mice that females express mate preferences for genetically dissimilar males (Roberts and Gosling 2003). Early studies with inbred congenic mouse lines showed that females chose males with major histocompatibility complex (MHC) types different from themselves, without prior experience (Penn and Potts 1998). Although manipulating artificial housing has revealed that MHC-dependent familial imprinting provides a more effective mechanism to avoid mating with kin, wild mice do not show behavioral imprinting on maternal MHC haplotypes (Penn and Potts 1998; Sherborne et al. 2007).

Olfactory cues are to some extent genetically determined. This odor–genes covariance means that odors emitted by rodents can be used by conspecifics to assess genetic relatedness

(Todrank and Heth 2003; Todrank et al. 2005). Closer genetic relationships between conspecifics are reflected by greater similarities in their odors and provide a basis for phenotype matching. Specifically, individuals with traits that most closely match an animal's template are its closest kin (Mateo 2003; Busquet and Baudoin 2005). Specific genotypes can be reflected by urine volatile composition in mice, and MHC may provide the main source of variation in odors used for individual recognition (Yamazaki et al. 1999). For example, MHC-determined urinary odor is composed of a mixture of volatile carboxylic acids relative to the characteristic of the odortype, though no genotype-unique compound has been detected (Singer et al. 1997). Modification of a single gene causes a significant change in the composition of urine-borne pheromones and elicits a distinct spatial pattern of glomerular activation within the main olfactory bulb (Schaefer et al. 2001; Novotny et al. 2007; Zhang et al. 2010). Volatile compounds comprising social odors can reflect genetic distances and relatedness in rodents and have been also found in interspecies (e.g., *Mus domesticus* and *M. spicilegus*), interstrain (e.g., ICR/CD-1, Kunming and C57B/6 mice), and family membership (e.g., beavers) (Sun and Müller-Schwarze 1998; Zhang, Rao, et al. 2007; Soini et al. 2009).

Rats (*Rattus norvegicus*) have also proven to be capable of discriminating between unfamiliar kin and nonkin via olfaction (Hepper 1983; Hepper 1991a). In particular, rats can distinguish among kin of different degrees of relatedness (Hepper 1987). Rats spend greater amount of time investigating less closely related conspecifics in the following order: cousins are investigated more than half siblings, which are investigated more than full siblings, and both unfamiliar and genetically unrelated rats are investigated most (Hepper 1987). Rats therefore possess a genetic identifier which is in direct proportion to their relatedness and can be used to discriminate degrees of kinship during phenotype matching (Hepper 1987). Such kin recognition may be primarily based on olfactory cues (Hepper 1983; Hopp et al. 1985). As in mice, voided urine, including volatile compounds derived in bladder urine and discharged from preputial glands, in rats serves as the major source of odor (Zhang, Liu, et al. 2008;Zhang, Sun, et al. 2008). However, little is known about genetically determined odor volatiles in this species, except for some work on pheromones or putative pheromones associated with gender, social hierarchy, pup–mother relations, and alarm (Brouette-Lahlou et al. 1991; Beynon and Hurst 2004; Gutiérrez-García et al. 2007; Pohorecky et al. 2008; Zhang, Sun, et al. 2008; Osada et al. 2009). We posit that genetically mediated volatiles of rats could provide olfactory information on genetic relatedness and function as a genetic identifier for phenotype matching.

The genes of inbred strains such as in mice are homozygous at nearly all loci, and the attraction of mice to the urinary odors of other mice is subject to a ''parent-of-origin'' effect which causes them to prefer the urine of unrelated strains to the same strain as their mothers (Beck et al. 2000; Isles et al. 2001). Here, we used 2 inbred strains of rats as a model to simulate kinship via genetic distance. We conducted combined binary choice tests and gas chromatography–mass spectrometry (GC-MS) to clarify whether kin recognition and inbreeding avoidance in rats is based on genetically determined odor similarity and dissimilarity. We used female Brown Norway (BN) rats of an inbred strain as odor recipients to investigate olfactory sex preferences for male urine of 2 inbred strains, BN and Lewis. We then used GC-MS to look for chemical differences between strains for subsequent use in behavioral experiments.

Materials and methods

Subjects

Ten male and 10 female BN rats and 8 male and 8 female Lewis rats were used as urine and preputial gland donors. Female BN rat donors were used as recipients. All animals were purchased at 20 weeks of age (Vital River Laboratory Animal Technology Co. Ltd). Males were housed individually and females in groups of 3–4 in plastic cages ($37 \times 26 \times 17$ cm). The room had a reversed 14:10 h light:dark photoperiod (lights on at 19:00) and was maintained at 23 ± 2°C. Food (standard rat chow) and water were provided ad libitum. We determined estrous cycles via vaginal smears for several days before behavioral testing and found that all females had an estrous cycle of 4 days, despite asynchrony; therefore, on a given test day, randomly selected recipient females would have covered all stages of the estrous cycle.

Scent collection and sample preparation

We placed rats in a clean mouse cage ($25 \times 15 \times 13.5$ cm) fitted with a wire grid 1 cm above the floor of the cage to collect urine for behavioral and chemical assay. Urine was absorbed immediately after it was voided using a disposable glass capillary and transferred to a vial in ice. Urine deposited next to feces was not collected. Animals were euthanized via cervical dislocation, and paired preputial glands were immediately dissected. Urine and preputial glands were individually sealed in vials and kept at –20 °C prior to use.

To characterize the composition of urine samples, we mixed 250 μL dichloromethane (purity >99.5%; DIMA Technology, Inc.) with 250 μL urine, stored it at 4 °C for 12 h, and then used the bottom phase (the layer with dichloromethane) for chemical analysis. To extract compounds from preputial gland secretion (PGS), the gland tissue was weighed, extracted in a volume of dichloromethane that reflected an extract concentration of 1 mg/3 μL solvent, and stored at 4 °C for 12 h. We then removed the tissue and used the remaining solution for GC-MS analysis.

In order to supplement the urine of each strain of male with putative signal compounds to simulate the other, we first diluted each compound with dichloromethane to a manageable concentration. We then transferred certain amounts of

solution to a clean vial, uncovered the vial, and allowed it to vaporize for 5 min. We then proportionally added the urine samples of one strain mixed equally from 10 males for BN rats or 8 males for Lewis rats into the vial to simulate the other. In detail, according to the results from the GC-MS analysis as described below, to simulate Lewis male urine, we added BN male urine with certain amounts of 4-heptanone, 2-heptanone, dimethyl sulfone, and 4-ethyl phenol to produce authentic concentrations equal to those in Lewis males, and we replenished BN urine with certain amounts of 4-heptanone and 2-heptanone to produce ratios equal to those in Lewis male urine. Likewise, dimethyl sulfone and 4-ethyl phenol were added into Lewis male urine to simulate BN male urine.

Behavioral assay

The responses of female BN rats to scented glass rods (20 cm long, 4-mm diameter) were measured in their home cages in a separate dim room during the dark phase of the light cycle (Lai et al. 1996; Zhang, Sun, and Novotny 2007; Zhang, Liu, et al. 2008). For each test, we kept one test rat in the home cage while temporarily moving its cage mates into an identical holding cage. One end of the glass rod was painted with 2 μL of urine; the other end was held by the tester. Two scented glass rods were simultaneously presented to a subject. We recorded the total time spent investigating for 3 min after the subject first sniffed or licked the rod tip (Zhang, Liu, et al. 2008). We repeated the test for each animal on another day and summed the investigation time over both trials for use.

For our habituation–dishabituation tests, we provided recipients with a urine sample from the same male 4 times and then introduced a novel sample on the fifth trial. We allowed 2 min between trials. We measured the time spent sniffing (within 1 cm of the rod) and licking each rod tip using stopwatches. To control for experimenter bias, the experimenter was blind to the nature of the sample. Using these methods, male urine samples of BN males were randomly paired with those of Lewis males and then presented to BN females. Each combination was used only once.

Gas chromatography–mass spectrometry analysis

We used an Agilent Technologies Network 6890N GC system coupled with 5973 Mass Selective Detector with the NIST/EPA/NIH Mass Spectral Library (2002 version; Agilent Technologies 2002). Xcalibur (Windows XP) was used for data acquisition and processing. The GC was equipped with a HP5-MS separation capillary column (30 m long, 0.25 mm inner diameter × 0.25-μm film thickness). Helium was used as the carrier gas (1.0 mL/min). The inject temperature was set at 280 °C. The oven temperature program was set initially at 50 °C, heated by 5 °C/min to 100 °C, then ramped by 10 °C/min until 280 °C, and held for 5 min. Finally, the temperature was increased to 300 °C and held for 10 min postrun to clean the column. Electron impact ionization was used at 70 eV. Transfer line temperature was set at 280 °C. Scanning mass ranged from 30–450 amu. We injected a 5 μL at a splitless mode for urine and 3 μL sample in a split mode (1:10) for PGSs.

Tentative identifications were made by comparing the mass spectra of GC peaks with those in the MS library (NIST2002). Thirteen of the tentatively identified compounds, 4-heptanone, 2-heptanone, dimethyl sulfone, 4-methyl phenol, 4-ethyl phenol, indole, *E*-β-farnesene, dodecanoic acid, tetradecanoic acid, hexadecanoic acid, Z9-octadecenoic acid, Octadecanoic acid, and squalene (all purity >98%; ACROS Organics) were further confirmed by matching retention times and mass spectra with the authentic analogs.

Abundance and relative abundance of compounds were used for quantitative comparisons between groups. The abundance of a particular compound was quantified by GC peak area. The peak area of a particular compound was then converted into a percentage of summed peak areas from all targeted GC peaks of either urine or PGS, as its relative abundance.

We quantified 4-heptanone, 2-heptanone, dimethyl sulfone, and 4-ethyl phenol in urine by comparing their GC areas in the samples with an established standard curve (GC area vs. concentration). To determine the variability of urine and PGS among individuals, we calculated relative standard deviation (RSD) using the formula: RSD = (standard deviation/mean) × 100, where the data we used for calculation were the percentage (relative abundances) of volatile peak areas for the 2 strains (Zhang, Rao, et al. 2007).

Statistical analysis

The distribution of raw data was examined using a Kolmogorov–Smirnov test and either parametric or nonparametric tests were applied to behavioral tests and GC data. If data were normally distributed, one-way analysis of variance with Bonferroni post hoc *t*-tests were used for GC data, whereas paired-samples *t*-test were used for behavioral data and RSDs. If data were not normally distributed, a Kruskal–Wallis *H* with post hoc Mann–Whitney *U* test was used for chemical data and Wilcoxon sign rank test for behavioral data and RSDs. All Statistical analyses were conducted using SPSS (v15.0, SPSS Inc.). Alpha was set at $P < 0.05$.

We used hierarchical cluster analysis to process GC-MS data from rat urine and PGS. Hierarchical cluster analysis is a statistical method for finding relatively homogenous clusters of cases based on measured characteristics. It sorts cases into clusters so that the degree of association is strong between members of the same cluster and weak between members of different clusters. We used this analysis (average linkage) with Pearson's correlation coefficient tests to examine the similarity of individual volatile profiles.

Ethical notes

The procedures of animal care and use in this study fully complied with Chinese legal requirements and were approved

by the Animal Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

Results

Preputial gland size

Preputial glands were found to be heavier (absolute weight) in males than females. The relative weight of the gland was heavier in Lewis females than males. Between strains, both the absolute and relative weight of the preputial gland were heavier for BN than Lewis males (Table 1).

Volatile composition of rat urine and PGS

We characterized 5 early eluting compounds from voided urine including 2 ketones, 1 sulfone and 2 phenols (Figure 1; Tables 2 and 3). We detected and identified 25 compounds from PGS including aldehydes, aliphatic acids, indole, and some terpenoid polyenes (Figure 2; Table 4).

The relative abundances of some volatile compounds within strains showed quantitative sexual dimorphism. For urine, 4-heptanone was found to be male-specific, 2-heptanone was higher in males, and dimethyl sulfone was higher in females in both BN and Lewis rats (Figure 1; Table 2). For PGS in BN rats, 8 compounds (3, 8, 9, 12, 20, 22, 23, and 25) were significantly higher and 5 compounds (2, 5, 6, 15, and 17) lower in males compared with females. Similarly for PGS in Lewis rats, 6 compounds (8, 9, 18, 20, 22, and 23) were significantly higher and 5 (2, 5, 6, 17, and 19) lower in males compared with females (Figure 2; Table 4).

Between strains and within gender, no strain-specific compounds in either urine or PGS were detected; but 4 compounds in urine and 9 compounds in PGS differed in relative abundance, quantified by percent GC areas, between BN and Lewis rats (Tables 2 and 4). In detail: 4-heptanone (Lewis vs. BN: 4.51% vs. 2.23%, $P = 0.021$, $z = 2.312$) and 2-heptanone (Lewis vs. BN: 76.05% vs. 43.31%, $P < 0.001$, $t = 7.549$) derived from male urine were significantly higher in Lewis male urine and dimethyl sulfone (Lewis: BN: 14.86% vs. 45.42%, $P = 0.001$, $z = 3.361$) and 4-ethyl phenol (Lewis:BN: 3.54%:8.77%, $P = 0.074$, $z = 1.785$, marginal significance) were higher in BN male urine than the other (Figure 1; Table 2). The PGS constituents 1, 7, and 14 were higher, but compounds 3, 8, 9, 12, 13, and 25 were lower in Lewis than in BN rats (Figure 2; Table 4). Abundance as reflected by GC peak area of 3 urine constituents exhibited considerable difference between the 2 strains (Table 3). In addition, the levels of 4-heptanone, 2-heptanone, dimethyl sulfone, and 4-ethyl phenol were estimated to be 0.26 ± 0.60, 2.86 ± 2.19, 4.68 ± 1.76, and 1.06 ± 0.55 ppm in BN male urine and 1.06 ± 0.90, 17.39 ± 6.56, 6.89 ± 2.12, and 1.47 ± 1.14 ppm in Lewis male urine, respectively.

We used cluster analysis to test the similarity of urine and PGS constituents among individual BN and Lewis males. A dendrogram of urine constituents reveals that the rats could be divided into 2 groups: a cluster composed of 9 individuals (8 Lewis and 1 BN rat); a cluster of 7 individuals (all BN). The cluster distance among Lewis rats was less than 5 (Figure 3). The dendrogram for PGS did not show such a classification (Figure 4).

Intrastrain variation in volatile compounds

In males of the 2 strains, the majority of volatile compounds in urine and PGS displayed extremely high interindividual RSDs. Most volatile compounds from the preputial gland showed higher interindividual than intraindividual RSDs, quantified by 6 duplicates of one sample (Tables 5 and 6).

Discrimination between individuals and preference between strains

Habituation–dishabituation tests showed that BN females habituated to repeated exposure to BN male urine ($P = 0.005$, $Z = 2.803$, $N = 10$). The time spent investigating the sample then increased when presented with a novel sample ($P = 0.005$, $Z = 2.805$, $N = 10$, Figure 5a).

Binary choice tests revealed that BN females were more attracted to BN male urine than to BN female urine ($P = 0.038$, $t = 2.073$, $N = 9$, Figure 5b) and that female BN rats showed a significant preference for Lewis male urine over BN male urine ($P = 0.001$, $t = 5.233$, $N = 9$, Figure 6a).

According to the authentic levels of urine constituents in 2 strains mentioned above, to simulate the rat urine of the other, 0.80 ppm 4-heptanone, 14.53 ppm 2-heptanone, 2.21 ppm dimethyl sulfone, and 0.41 ppm 4-ethyl phenol were added to BN male urine so that levels were equal to those of Lewis males. After replenishing the male urine with

Table 1 Comparison of the weights of body and preputial gland (PG) between sexes and strains of rats (mean ± standard deviation)

Items	Groups			
	BN males ($n = 6$)	BN females ($n = 6$)	Lewis males ($n = 8$)	Lewis females ($n = 8$)
Body weight (BW) (g)	$318.97 \pm 18.65^{a,b}$	192.00 ± 15.39^{b}	$475.40 \pm 14.57^{a,c}$	250.94 ± 25.47^{c}
PG weight (mg)	$128.25 \pm 21.08^{a,b}$	87.62 ± 23.32^{b}	$115.49 \pm 23.84^{a,c}$	89.10 ± 20.03^{c}
Relative PG weight (mg/100 g BW)	40.25 ± 6.67^{a}	45.25 ± 9.36	$24.30 \pm 4.98^{a,c}$	35.57 ± 7.38^{c}

The means in a row marked by the same superscript letters show significant differences ($P < 0.05$, using one-way ANOVA with Bonferroni post hoc *t*-test).

synthetic analogs, we found that BN females responded equally to pure BN male urine and adjusted BN male urine (P = 0.150, t = 1.591, N = 9). Meanwhile, Lewis male urine was also more attractive than adjusted BN male urine (P = 0.038, t = 2.073, N = 9, Figure 6a).

Because of interstrain differences in the ratio of the 4 compounds, we needed to supplement the urine from each strain of male. We added 2-heptanone and 4-heptanone to BN male urine to simulate Lewis male urine using the formula:

$$\begin{cases} \dfrac{2.23\% + x}{1 + x + y} = 4.51\% \\ \dfrac{43.31\% + y}{1 + x + y} = 76.05\% \end{cases}$$

where, x and y represented the percentage of 4-heptanone and 2-heptanone added to BN male urine. Therefore, 1.21 ppm 4-heptanone and 11.21 ppm 2-heptanone were added to BN male urine, increasing the proportion of 4-heptanone and 2-heptanone to 4.51% and 76.05%, respectively.

Similarly, when adding dimethyl sulfone and 4-ethyl phenol to Lewis male urine, to make it similar to BN male urine, we let x and y be the percentage of dimethyl sulfone and 4-ethyl phenol added to Lewis male urine using the formula:

$$\begin{cases} \dfrac{14.86\% + x}{1 + x + y} = 45.42\% \\ \dfrac{3.54\% + y}{1 + x + y} = 8.77\% \end{cases}$$

Consequently, 30.62 ppm dimethyl sulfone and 5.02 ppm 4-ethyl phenol were added to Lewis male urine so that their ratio was equal to those in BN male urine (Tables 2 and 3).

Further 2-choice tests showed that BN females responded equally to Lewis male urine and BN male urine following supplementation (P = 0.655, t = 0.465, N = 9). No preference was found between BN male urine and Lewis male urine supplemented with dimethyl sulfone and 4-ethyl phenol (P = 0.415, t = 0.678, N = 9). The attractiveness of BN male urine was significantly increased after supplementation (P = 0.015, t = 2.429, N = 9), and Lewis male urine was more attractive than Lewis male urine following supplementation (P = 0.051, t = 1.955, N = 9). Moreover, females showed a significant preference for supplemented BN male urine over Lewis male urine (P = 0.015, t = 2.429, N = 9, Figure 6b).

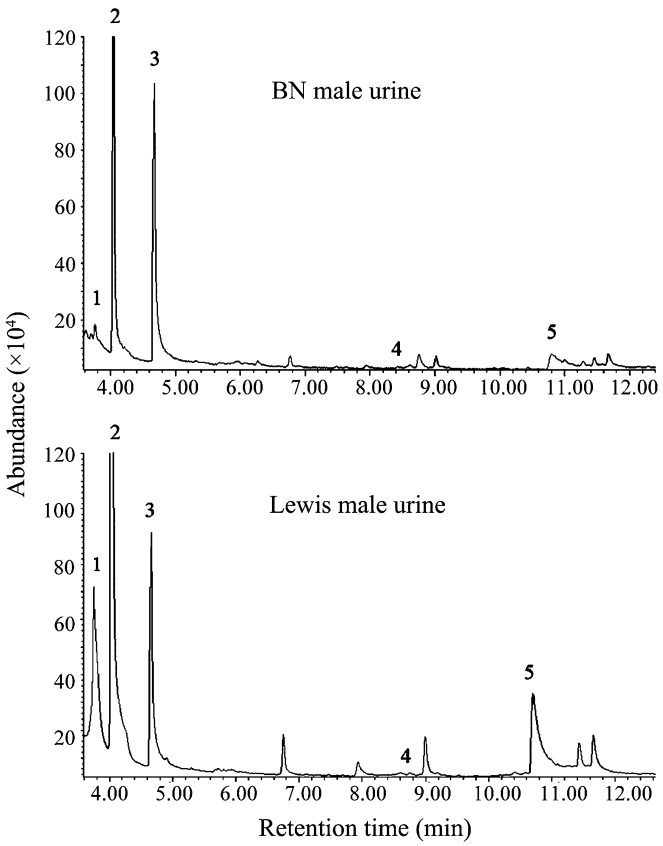

Figure 1 Representative GC profile of dichloromethane extract from male urine. GC conditions are described in Materials and methods section. Numbered GC peaks correspond to compounds in Tables 2 and 3. Peak 1, 2, 3, 5 are 4-heptanone, 2-heptanone, dimethyl sulfone and 4-ethyl phenol, respectively.

Table 2 Comparison of the relative abundance of urinary volatiles between groups (mean ± standard deviation, n = 8 for each group)

GC peak	RT (min)	Compounds	BN		Lewis		Six duplicate of one sample
			Male	Female	Male	Female	
1	3.71	4-Heptanone*	2.23 ± 4.21^{a,b}	0.00 ± 0.00^{b}	4.51 ± 2.96^{a,c}	0.00 ± 0.00^{c}	2.57 ± 0.15
2	4.00	2-Heptanone*	43.31 ± 10.22^{a,b}	9.26 ± 11.56^{b}	76.05 ± 6.77^{a,c}	2.29 ± 0.68^{c}	69.01 ± 1.73
3	4.66	Dimethyl sulfone*	45.42 ± 12.95^{a,b}	76.48 ± 18.09^{b}	14.86 ± 4.50^{a,c}	93.17 ± 4.34^{c}	11.41 ± 0.80
4	8.35	4-Methyl phenol*	0.27 ± 0.76	0.00 ± 0.00	1.05 ± 2.89	0.04 ± 0.11	5.84 ± 0.71
5	10.70	4-Ethylphenol*	8.77 ± 7.23	14.26 ± 14.54	3.54 ± 4.30	4.50 ± 4.21	11.17 ± 0.55

The means in a row marked by the same superscript letters show significant differences (P < 0.05, using one-way ANOVA with Bonferroni post hoc t-test or Kruskal–Wallis H with post hoc Mann–Whitney U test). The compound marked by asterisks are definitively identified. RT, retention time.

Table 3 Comparison of the abundance of urinary volatiles (mean ± standard deviation, $n = 8$ for each group)

GC peak	RT (min)	Compounds	BN		Lewis		Six duplicate of one sample
			Male	Female	Male	Female	
1	3.71	4-Heptanone*	23.52 ± 54.78[a,b]	0.00 ± 0.00[b]	104.74 ± 88.39[a,c]	0.00 ± 0.00[c]	93.99 ± 8.60
2	4.00	2-Heptanone*	262.13 ± 218.56[a,b]	21.39 ± 23.13[b]	1711.17 ± 655.89[a,c]	5.47 ± 2.21[c]	2528.64 ± 250.47
3	4.66	Dimethyl sulfone*	211.04 ± 87.89[a]	207.63 ± 111.12	321.62 ± 105.98[a]	219.38 ± 44.94	418.28 ± 50.30
4	8.35	4-Methyl phenol*	2.06 ± 5.77	0.00 ± 0.00	32.58 ± 90.71	0.08 ± 0.21	215.46 ± 42.46
5	10.70	4-Ethylphenol*	51.02 ± 60.90	34.47 ± 30.77	95.80 ± 126.11	11.34 ± 11.77	410.32 ± 53.44

The means in a row marked by the same superscript letters show significant differences ($P < 0.05$, using one-way ANOVA with Bonferroni post hoc *t*-test or Kruskal–Wallis *H* with post hoc Mann–Whitney *U* test). The compound marked by asterisks are definitively identified. RT, retention time.

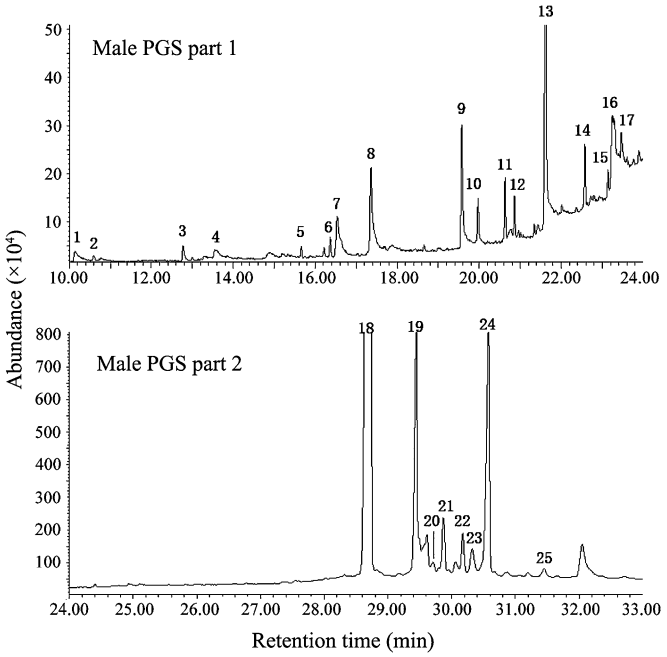

Figure 2 Representative GC profile of dichloromethane extract from male preputial gland (top: 10–24 min; bottom: 24–33 min). GC conditions are described in Materials and methods section. Numbered GC peaks correspond to compounds in Table 4. Peak 5, 6, 8, 9, 10, 13, 18 are *E*-β-farnesene, *E,E*-α-farnesene, dodecanoic acid, tetradecanoic acid, hexadecanal, hexadecanoic acid, and squalene, respectively.

Discussion

Previous behavioral work suggests a correlation between genes and social odor and that odor can be used by conspecifics during olfactory assessment of genetic relatedness by rodents (Hepper 1983; Hepper 1991a; Todrank and Heth 2003; Todrank et al. 2005). It therefore appears that rats can distinguish among kin of different relatedness (Hepper 1987). Inbred mice strains are approximately identical in genotype and homozygous at nearly all loci (Beck et al. 2000). The similarity in volatile composition of individual scent reflects this genetic similarity (Singer et al. 1997; Beauchamp and Yamazaki 2003). Here, we used 2 inbred strains of rats as a model to simulate kinship and showed that inbred BN female rats had a normal preference for male urine over female urine of their own strain (Figure 5b) and chose Lewis male urine over BN male urine (Figure 6a). Because female recipients represented all stages of the estrous cycle, we do not think that endocrine state affected female behavior in our experiments. That is, we posit that females show a preference for a specific odor regardless of their estrous state. Our findings suggest that urine-borne volatiles are capable of conveying olfactory cues to females to assess genetic relationships during mate choice and avoid inbreeding. Much evidence shows that animals avoid breeding with close relatives (Pusey and Wolf 1996) and that females may ensure the heterozygosity of their offspring via odortype matching when choosing mates (Brown 1997). These results from inbred female rats support the "good-genes-as-heterozygosity" hypothesis that females, especially inbred females, may choose mates that are genetically dissimilar and result in offspring of greater heterozygosity (Isles et al. 2001; Ilmonen et al. 2009). Inbred females may gain more benefits from this strategy than outbred females (Ilmonen et al. 2009).

Chemically, we characterized 30 compounds from urine and PGS of BN and Lewis rats and found neither strain-specific nor sex-specific compounds, except male-specific 4-heptanone. However, quantitatively, 2-heptanone was richer in males than in females, and dimethyl sulfone and 2 preputial gland–secreted sesquiterpens, *E*-β-farnesene and *E,E*-α-farnesene, were richer in females (Figures 1 and 2, Tables 2, 3, and 4). These results are consistent with those previously reported in Sprague-Dawley rats (Zhang, Sun, et al. 2008; Osada et al. 2009) and suggest that rats of different strains may share similar odor volatiles coding for chemical signals. Moreover, the volatiles are species shared. Some of these compounds such as 2-heptanone, dimethyl sulfone, and/or 4-heptanone have also been found in the urine of other mammals including minks, dogs, and rabbits (Zhang YH, Zhang JX, unpublished data).

Table 4 Comparison of relative abundance of preputial gland volatiles between groups (mean ± standard deviation, $n = 6$ for each group)

GC peak	RT (min)	Compounds	BN		Lewis		Six duplicate of one sample
			Male	Female	Male	Female	
1	10.26	Unknown	0.01 ± 0.02[a]	0.01 ± 0.02	0.05 ± 0.03[a]	0.06 ± 0.02	0.03 ± 0.01
2	10.67	Unknown	0.01 ± 0.01[b]	0.05 ± 0.02[b]	0.01 ± 0.01[c]	0.11 ± 0.07[c]	0.02 ± 0.01
3	12.83	3,7-Dimethyl-2,6-Octadienal	0.08 ± 0.01[a,b]	0.04 ± 0.01[b]	0.05 ± 0.01[a]	0.06 ± 0.01	0.04 ± 0.00
4	13.67	Indole*	0.02 ± 0.02	0.01 ± 0.01	0.04 ± 0.02	0.03 ± 0.04	0.03 ± 0.02
5	15.70	*E*-β-farnesene*	0.03 ± 0.01[b]	0.05 ± 0.02[b]	0.02 ± 0.01[c]	0.08 ± 0.03[c]	0.02 ± 0.01
6	16.40	*E,E*-α-farnesene*	0.05 ± 0.02[b]	0.10 ± 0.04[b]	0.04 ± 0.01[c]	0.18 ± 0.06[c]	0.04 ± 0.01
7	16.59	Unknown	0.06 ± 0.04[a]	0.06 ± 0.05	0.19 ± 0.05[a]	0.17 ± 0.07	0.09 ± 0.01
8	17.45	Dodecanoic acid*	0.61 ± 0.22[a,b]	0.27 ± 0.06[b]	0.36 ± 0.08[a,c]	0.15 ± 0.04[c]	0.31 ± 0.07
9	19.65	Tetradecanoic acid*	0.75 ± 0.24[a,b]	0.40 ± 0.12[b]	0.33 ± 0.03[a,c]	0.07 ± 0.02[c]	0.34 ± 0.07
10	20.02	Hexadecanal	0.15 ± 0.12	0.19 ± 0.11	0.07 ± 0.04	0.30 ± 0.28	0.09 ± 0.03
11	20.68	Octadecenal	0.03 ± 0.01	0.02 ± 0.01	0.06 ± 0.03	0.05 ± 0.05	0.04 ± 0.00
12	20.89	A terpenoid polyene	0.11 ± 0.03[a,b]	0.04 ± 0.01[b]	0.07 ± 0.02[a]	0.05 ± 0.01	0.05 ± 0.00
13	21.70	Hexadecanoic acid*	1.48 ± 0.30[a]	1.55 ± 0.14	1.07 ± 0.17[a]	1.32 ± 0.35	1.05 ± 0.20
14	22.63	2-pentadecyl-1,3-Dioxolane	0.01 ± 0.01[a]	0.02 ± 0.02	0.06 ± 0.03[a]	0.06 ± 0.03	0.04 ± 0.01
15	23.19	Palmidrol	0.04 ± 0.02[b]	0.11 ± 0.04[b]	0.05 ± 0.02	0.09 ± 0.04	0.04 ± 0.00
16	23.36	Z9-octadecenoic acid*	0.52 ± 0.14	0.61 ± 0.11	0.39 ± 0.14	0.53 ± 0.09	0.53 ± 0.06
17	23.54	Octadecanoic acid*	0.07 ± 0.03[b]	0.12 ± 0.03[b]	0.06 ± 0.02[c]	0.13 ± 0.07[c]	0.07 ± 0.01
18	28.78	Squalene*	64.71 ± 2.73	65.71 ± 2.13	66.75 ± 1.20[c]	64.16 ± 2.16[c]	70.27 ± 1.72
19	29.52	A terpenoid polyene	10.23 ± 1.21	11.92 ± 1.38	10.75 ± 1.87[c]	13.56 ± 1.22[c]	9.62 ± 0.77
20	29.80	A terpenoid polyene	0.28 ± 0.06[b]	0.16 ± 0.06[b]	0.29 ± 0.14[c]	0.08 ± 0.04[c]	0.31 ± 0.03
21	29.96	A terpenoid polyene	2.38 ± 0.17	2.32 ± 0.17	2.34 ± 0.20	2.45 ± 0.07	2.42 ± 0.08
22	30.27	A terpenoid polyene	1.72 ± 0.23[b]	1.40 ± 0.19[b]	1.48 ± 0.14[c]	1.12 ± 0.27[c]	1.35 ± 0.11
23	30.43	A terpenoid polyene	1.23 ± 0.33[b]	0.33 ± 0.08[b]	1.15 ± 0.41[c]	0.28 ± 0.09[c]	0.55 ± 0.05
24	30.70	A terpenoid polyene	14.43 ± 1.36	14.12 ± 0.94	13.67 ± 0.96	14.51 ± 0.34	12.22 ± 0.66
25	31.56	A terpenoid polyene	1.00 ± 0.21[a,b]	0.40 ± 0.07[b]	0.65 ± 0.31[a]	0.42 ± 0.06	0.45 ± 0.04

The means in a row marked by the same superscript letters show significant differences ($P < 0.05$, using one-way ANOVA with Bonferroni post hoc *t*-test or Kruskal–Wallis *H* with post hoc Mann–Whitney *U* test). The compound marked by asterisks are definitively identified. RT, retention time.

Pairwise comparison revealed that many volatiles differed significantly between strains (Tables 2, 3, and 4). Moreover, the dendrogram from the cluster analysis revealed that urine-derived volatiles could better reflect genetic similarity and dissimilarity than PGS-produced volatiles in rats and that this similarity may reflect a closer genetic relationship (Figures 3 and 4). As for urine-derived volatiles, individuals shared more similarities within strain than between strain (Figure 3). As in mice, urine-derived volatiles are more sensitive to genetic shifts than PGS-produced volatiles (Zhang, Rao, et al. 2007). Some scent volatiles have been demonstrated to be capable of coding for olfactory genetic information in mice (Singer et al. 1997; Schaefer et al. 2001; Novotny et al. 2007). Our pairwise comparison revealed that urine-derived 4-heptanone, 2-heptanone, dimethyl sulfone, and 4-ethyl phenol differed in relative and/or absolute abundance for BN and Lewis males and might code for such genetic information in voided urine. Singer et al. (1997) demonstrated that urinary volatiles covary in relative concentrations with genotypes and contribute to unique individual odors (odortypes) in mice. The relative concentration or ratio of urine components is a vital index for putative chemical signal components and our results also support this notion.

Replenishing BN male urine with 4-heptanone, 2-heptanone, dimethyl sulfone, and 4-ethyl phenol at absolute levels similar

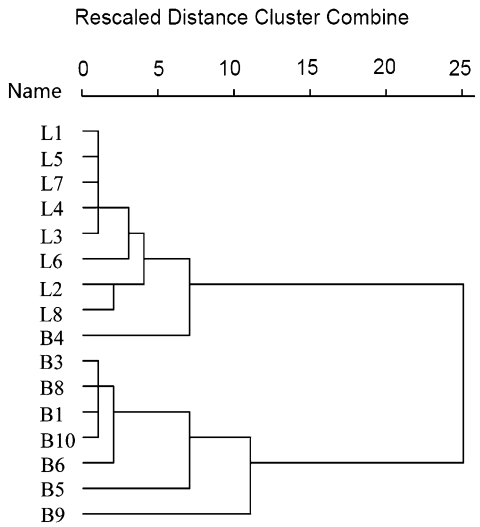

Figure 3 Dendrogram for average linkage hierarchical clustering of urine data (the relative abundances of volatile compounds for BN and Lewis male rats). Each sample is represented by the initial letter of the strain name affixed with a number (L stands for Lewis male urine and B stands for BN male urine, eight individuals in each group).

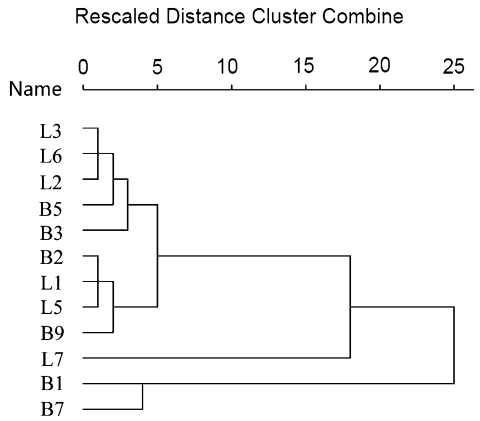

Figure 4 Dendrogram for average linkage hierarchical clustering of PGS data (the relative abundances of volatile compounds for BN and Lewis male rats). Each sample is represented by the initial letter of the strain name affixed with a number (L stands for Lewis male PGS and B stands for BN male PGS, six individuals in each group).

to Lewis male urine did not result in a difference in BN female response (Figure 6a). Although we have regulated all between-strain different compounds of BN male urine to Lewis male urine levels, the responses of BN females remained the same. Because variation in the relative abundance of the 4 compounds proved to determine the chemical signals between strains, we speculate that 4-methyl phenol and other GC-undetected compounds in urine might affect the ratio of the 4 compounds in spiked BN male urine. This meant that they could not reach the ratios exactly as those present in Lewis male urine.

Taking relative concentration into consideration, 2-heptanone and 4-heptanone were found at a higher ratio

Table 5 Individual variation (RSD) of relative abundance of the urine volatiles of 2 strains of rats

GC peak	BN	Lewis	Six duplicate of one sample
1	188.24	65.66	5.98
2	23.61	8.91	2.50
3	28.52	30.29	6.98
4	281.27	276.56	12.12
5	82.52	121.45	4.88
Mean ± SD	120.83 ± 111.50	100.58 ± 107.20	6.49 ± 3.56
Z values	2.023	2.023	
P values	0.043	0.043	

RSD was calculated using the formula RSD = (standard deviation/mean) × 100, where mean and standard deviation (SD) are the average of each volatile peak area (in percentage) and their SD, respectively. Wilcoxon matched-pair signed-rank test for RSDs between same compounds of each individual group.

in Lewis males, whereas dimethyl sulfone and 4-ethyl phenol were higher in BN males. These compounds may form the main part of the odortype for these strains. We focused on only those constituents which had lower relative ratios and spiked BN and Lewis male urine with synthetic analogs. The ratios of 2-heptanone and 4-heptanone in spiked BN urine were the same as those for Lewis, but the ratios of dimethyl sulfone and 4-ethyl phenol were changed and not equal to either BN or Lewis. However, individual variation in BN urine does exist (mean dimethyl sulfone abundance was 14.86% ± 4.50%, 4-ethyl phenol was 3.54% ± 4.30%), so our simulation should be acceptable. Furthermore, although the ratios of 4-heptanone and 2-heptanone were changed, spiked Lewis male urine was similar to BN male urine. We therefore succeeded in simulating BN male urine through the addition of both dimethyl sulfone and 4-ethyl phenol to Lewis male urine, and BN females preferred pure Lewis male urine over rescented urine from Lewis males. Similarly, we successfully simulated Lewis male urine by adding 4-heptanone and 2-heptanone to BN male urine. BN females did not differ in their response for pure BN male urine and rescented Lewis male urine and showed a preference for rescented BN male urine over rescented Lewis male urine (Figure 6b). Behavioral tests revealed that these preferences were based on preferences for another strain rather than preference for novel stimuli (if it were because of novel stimuli, preference should always have been for spiked urine). Hence, our data show that the relative concentration of scent volatile compounds may be reliable indicators when screening for potential genetically determined chemosignals.

In addition, the large interindividual variation of volatile compounds detected in rat urine and PGS indicates that urine-borne volatiles might code for individual information in the analog form, as in mice (Zhang, Rao, et al. 2007).

Table 6 Individual variation (RSD) of relative abundance of the preputial gland volatiles of 2 strains of rats

GC peak	BN		Lewis		Six duplicate of one sample
	Male	Female	Male	Female	
1	237.10	244.95	57.19	38.39	17.07
2	74.42	32.79	53.44	62.46	32.91
3	15.20	17.70	24.36	12.82	9.49
4	95.96	114.92	47.23	107.59	48.43
5	41.02	32.20	28.38	34.27	25.26
6	42.66	34.83	34.28	31.69	29.58
7	69.84	96.59	29.03	37.98	9.97
8	35.72	21.14	21.51	24.55	22.30
9	32.51	29.65	9.72	35.35	19.15
10	76.45	60.79	58.63	93.11	32.27
11	40.21	48.00	54.56	93.44	9.12
12	25.67	34.59	33.49	25.76	10.10
13	20.10	8.87	15.79	26.90	18.67
14	92.20	125.55	54.37	50.89	21.37
15	43.95	38.33	35.90	47.51	7.71
16	26.93	17.55	34.86	16.40	11.73
17	41.56	22.86	38.01	56.04	21.33
18	4.22	3.24	1.79	3.37	2.45
19	11.83	11.61	17.43	9.02	8.01
20	23.19	37.14	49.30	54.16	9.16
21	7.17	7.52	8.61	2.85	3.32
22	13.58	13.30	9.62	24.33	8.06
23	27.04	24.04	36.14	31.09	9.77
24	9.45	6.63	7.05	2.34	5.44
25	20.91	16.73	47.98	14.07	8.75
Mean ± SD	45.16 ± 47.67	44.06 ± 52.82	32.35 ± 17.59	37.46 ± 28.40	16.06 ± 11.10
P values	0.000	0.000	0.000	0.000	
Z values	4.372	3.888	3.754	4.184	

RSD was calculated using the formula RSD = (standard deviation/mean) × 100, where mean and standard deviation (SD) are the average of each volatile peak area (in percentage) and their SD, respectively. Wilcoxon matched-pair signed-rank test for RSDs between same compounds of each individual group.

Specifically, the information could be coded by varying amounts of shared compounds rather than by unique compounds. In habituation–dishabituation tests, rats were capable of discriminating 2 odor stimuli, suggesting that the urine odor of the extremely inbred BN rats does differ between individuals (Figure 5a). In agreement with behavior tests, the high interindividual divergence in volatile composition as reflected in high RSDs (Tables 5 and 6) may lay the foundation for learned individual recognition or memorization of individuals via urine odor and provide information about individual genotypes despite intrastrain similarities in genotypes and odortypes of inbred rats. Indeed, previous results from mice have shown that small changes in genotype (e.g., MHC genes or Foxn1 gene) cause significant change in the composition of urine volatiles and consequently change the responses of recipient mice (Singer et al. 1997; Schaefer et al. 2001; Novotny et al. 2007; Keller et al. 2009; Zhang et al. 2010).

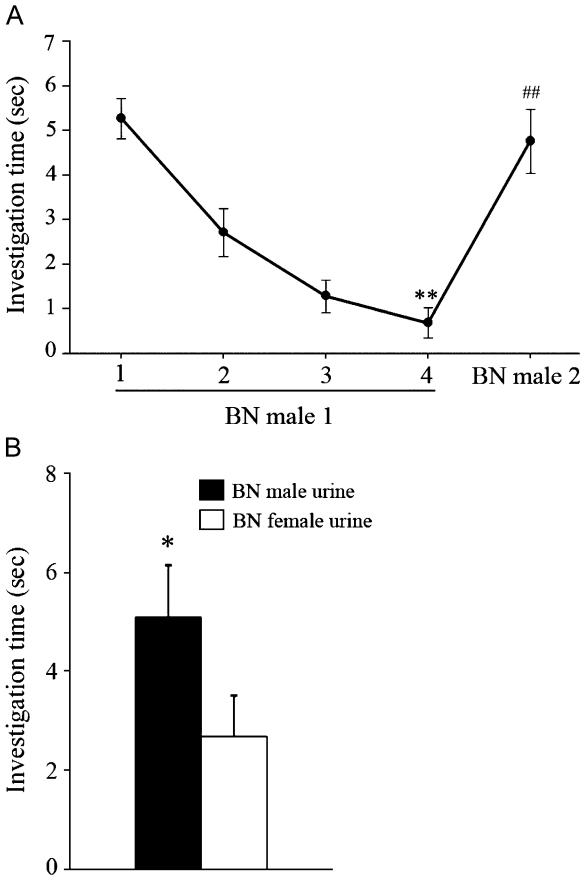

Figure 5 **(a)** Duration of investigation (mean ± standard error, s) by female BN rats of male and female BN rat urine samples during a 3 min choice test (* , $P < 0.05$, paired *t*-test). **(b)** Discrimination of BN female rats between different BN male urine samples. Investigation time of the fourth presentation was lower compared to the first presentation (**, $P < 0.01$, Wilcoxon matched-pairs signed-rank test). Investigation time during the test presentation increased compared with the fourth presentation of the the habituated sample (##, $P < 0.01$, Wilcoxon matched-pairs signed-rank test).

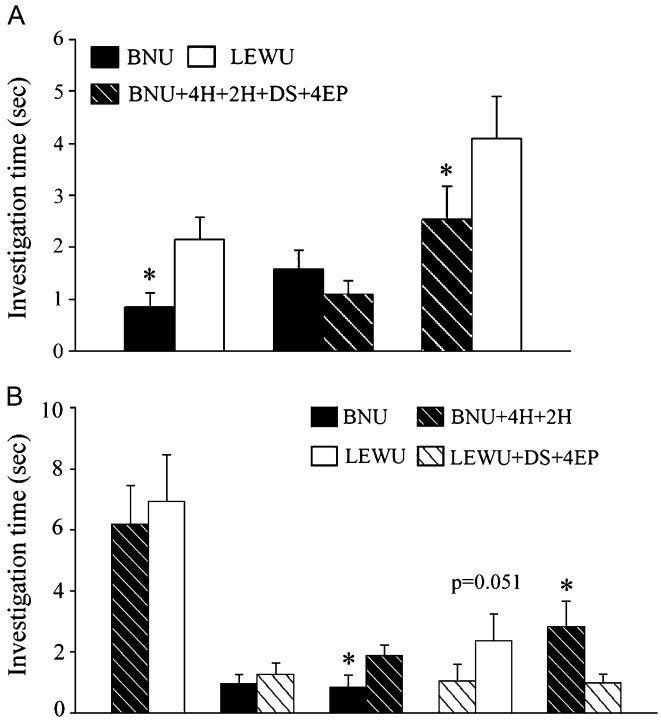

Figure 6 Duration of investigation (mean ± standard error, s) by female BN rats of different male urine samples during a 3 min choice test across two days. **(a)** Synthetic analogues added to BN urine according to authentic levels present in Lewis male urine. **(b)** Synthetic analogues added to male urine according to relative abundance in each of the other strains (*, $P < 0.05$, paired *t*-test or Wilcoxon matched-pairs signed-rank test).

In conclusion, female rats may use olfactory cues to assess relatedness in potential mates and choose mates with odortypes different from their own during mate choice and inbreeding avoidance. In particular, urine-derived volatiles such as 4-heptanone, 2-heptanone, dimethyl sulfone, and 4-ethyl phenol show covariation between relative abundance and degree of genetic relatedness, and these compounds may play a key role in chemical signaling, genetic identity, and kin recognition in the rat.

Funding

This research was supported by the National Basic Research Program of China (973 Program, grant 2010CB833900-2); State Key Laboratory of Integrated Management of Pest Insects and Rodents in Agriculture (Chinese IPM0906); and Chinese National Science Foundation (grants 30670268, 30870297).

Acknowledgements

We are grateful to Jin-Hua Zhang for assistance with behavioral tests and Xiaowei Qin for assistance with the GC-MS.

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