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Original Article

Species recognition and the divergences in the chemical and ultrasonic signals between two coexisting *Rattus* species

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Abstract

The ability to recognize and differentiate between conspecifics and heterospecifics as well as their signals is critical for the coexistence of closely related species. In the genus *Rattus*, species are morphologically similar and multiple species often coexist. Here, we investigated the interspecific recognition and signal differentiation of two sympatric rat species, the brown rat (*Rattus norvegicus*, RN) and the Asian house rat (*Rattus tanezumi*, RT). In a two-way choice test, both RN and RT females showed a preference for conspecific male rats to heterospecific ones. RT females showed a significant preference for accessible urine of males of same species to those of other species, but not for the inaccessible urine. On the other hand, there were significant differences in the structural characteristics of the ultrasonic vocalization emitted by males of these two rat species. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing electrophoresis unveiled that major urinary proteins (MUPs) in voided urine were more highly expressed in RN males versus RT males. The interspecific differences of urinary volatile compounds were also discussed. In conclusion, female rats had the ability to distinguish between males of either species.

Key words: Asian house rats, brown rats, closely related species, pheromones, ultrasonic vocalization.

Species recognition or discrimination plays an important role in premating isolation and maintaining species boundaries in closely related sympatric species (Mallet 1995; Panhuis et al. 2001; Svensson et al. 2007; M'Gonigle et al. 2012). In the process of interspecific discrimination, an individual's behavior is largely governed by the signals or cues emitted by other individuals of the same or a different species (Kaur et al. 2014). The differentiation of interspecific signals between coexisting closely related species facilitates interspecific discrimination and reduces interspecific mismating and unnecessary physical interference (Boughman 2002; Gröning and Hochkirch 2008; Anderson and Grether 2010; Zhang et al. 2013; Varner et al. 2020). To reduce reproductive interaction, resource competition, and recognition of conspecifics as rivals caused by interspecific misidentification among closely related species, the differences in certain traits, such as communication signals, are accentuated to reinforce interspecific discrimination when the species coexist (i.e. character displacement) (Higgie et al. 2000; Pfennig and Pfennig 2009; Stuart and Losos 2013). In brief, the interspecific recognition is essential for coexistence of closely related species and is often based on the interspecific differentiation of communication signals.

The genus *Rattus* is the most diverse genus of rodents, with 66 species, which evolved fairly recently but do not exhibit overt ecomorphological divergence among species (Musser and Carleton 2005; Rowe et al. 2011). *Rattus norvegicus* (RN) and *Rattus tanezumi* (RT) are closely related species, highly similar in morphology and niche, and are sympatric in many parts of China (Guo et al. 2017; Chen et al. 2021; Jing et al. 2022). About 30 years ago, RT rats had spread from southern China, across the Yellow River, into North China, where their ranges began to overlap with those of the North China subspecies of *R. n. humiliates* (RNH) and often coexist and share habitats (Zhang et al. 2000; Chen et al. 2021). Invasive species tend to be more aggressive than native species, but individuals of different species do not fight as fiercely as individuals of the same species in both RN and RT, thus, interspecies recognition is required to regulate the interspecies relationship between these two rat species (Guo et al. 2017; Chen et al. 2021). These two coexisting rat species could serve

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as a model for studying species recognition and divergences of communication signals among all *Rattus* species with physical resemblance.

Chemical communication is considered one of the most common communication modalities in animals (Wyatt 2014). Chemical signals can be deposited in the environment and function for a long time in the absence of signalers (Córdoba-Aguilar et al. 2018; Varner et al. 2020). The importance of chemical communication in species recognition has been shown in closely related sympatric species of fish, salamanders, lizards, rodents, and insects (Dawley 1984; Singer 1998; McLennan and Ryan 1999; Todrank and Heth 2003; Barbosa et al. 2006; Rafferty and Boughman 2006). Pheromones as the signals in intraspecific chemical communication are compounds released by one organism that modulate the behavior or physiology of a conspecific and share some main compounds with allomones as the signals in interspecific chemical communication (Papes et al. 2010; Wyatt 2014; Zhang et al. 2016). Pheromones of closely related species with a sympatric distribution may lead to significant interspecific differences due to character displacement, prompting accurate interspecific identification and likely resulting in altered intraspecific pheromone function (Higgie et al. 2000; Pfennig and Pfennig 2009; Stuart and Losos 2013).

In rodents, pheromones and/or allomones are usually produced in urine, specialized skin glands and lacrimal glands, and their variation may drive species divergence and play an important role in premating isolation between species and maintaining species boundaries (Smadja and Butlin 2009; Brennan 2010). The compounds used as mammal pheromones include small volatile molecules, steroid derivatives, peptides, and large protein–ligand complexes (Liberles 2014; Wyatt 2014). In rats, 2-heptanone, 4-heptanone, 9-hydroxy-2-nonanone, major urinary protein 13 (MUP13), and OBP3 have been identified as male pheromones (Zhang and Zhang 2014; Zhang et al. 2008a; Guo et al. 2019). Our previous research showed that organic volatile composition in urine is differentiated between RN males and RT males (Zhang and Zhang 2014; Guo 2016) (unpublished data). MUPs can also serve as contact pheromones and allomones to mediate intra- and interspecific interaction (Hurst et al. 2001; Papes et al. 2010; Roberts et al. 2010). To understand the interspecific recognition processes of these two closely related rat species, it is necessary to compare their urine-borne volatiles and MUPs.

Species recognition is a complex process involving communication between potential partners, using olfactory, auditory, and/or visual cues (Ben-Shaul et al. 2010; Dorado-Correa et al. 2013; Cervo et al. 2015). Divergent signaling traits between species individually or jointly contribute to species recognition (Panhuis et al. 2001; Laidre and Johnstone 2013). In addition to above-mentioned chemical signals, acoustic signals including audible sound, ultrasound, and infrasound, also play a variety of roles in behavioral interactions between individuals of the same species and different species in most animal groups (Gerhardt et al. 2003; Charlton and Reby 2016). Pheromones emitted by male mice might elicit the initial responses of female partners, leading to further actions such as ultrasonic vocalizations (USVs) by females and behavioral interactions such as approaching and mating (Demir et al. 2020). Rats produce USVs in a variety of frequency ranges to communicate their affective state in social situations, where 50-kHz USVs and 22-kHz USVs communicate positive and negative affective states, respectively, to conspecific receivers (Wöhr and Schwarting 2013; Inagaki and Ushida 2021). USVs also exhibit significant divergences among rat (*Rattus*) or mouse (*Mus*) species and may therefore be involved in species recognition (Musolf et al. 2015; Chen et al. 2017). Since effective visual communication is based on apparent differences in the appearance of visual signals, visual communication may not be as effective as chemical and acoustic communication in interspecific discrimination of the genus *Rattus* rat species looking very similar in appearance (Rowe et al. 2011; Caro and Allen 2017).

The diverged acoustic signals and chemical signals have been demonstrated to be particularly useful for species recognition between coexisting similar species in many mammal groups, but they have been seldom examined among coexisting similar rat species of the genus *Rattus* (Heth et al. 1999; Perri and Randall 1999; Łopucki and Szymroszczyk 2003; Johnston and Robinson 2010; Guo et al. 2017; Chen et al. 2017; Apps et al. 2019; Varner et al. 2020). Here, we hypothesized that the chemical signals and/or USV signals of males were divergent between RN and RT rat species if female rats were able to distinguish between conspecific and heterospecific male rats with physical resemblance.

Materials and Methods

Subjects

The ancestors of RN rats used were captured in Beijing, North China, and the ancestors of RT rats were captured in Taiyuan City, Shanxi Province, North China, in the summer of 2010. Each species was maintained as a closed-outbred colony in our laboratory. Paired male and female subjects of the same species in the two-choice tests were from different parents and litters and were strangers to each other. The rats used here were the tenth generation produced under these laboratory conditions. After being weaned at four weeks of age, the rats were kept in same-sex sibling groups in plastic rat cages (37 × 26 × 17 cm) (Suzhou Feng's Laboratory Animal Equipment Co., Ltd., Suzhou, China) (14:10 h light/dark cycle, lights on at 19:00) with wood shavings for bedding (Beijing Keao Xieli Feed Co., Ltd., Beijing, China) at 23 ± 2 °C. Standard rat chow and water were provided ad libitum. The age of all rats was 5–12 months. The animal handling procedure complied with the guidelines of the Animal Use Committee of the Institute of Zoology, Chinese Academy of Sciences (IOZ 2022).

Urine collection

Urine was collected from 6 males of each subspecies. The age of all male rats was 5–12 months. The rats were individually caged in clean metabolic cages for 8 h per day during the dark phase of the light/dark cycle. Standard rat chow and water were provided ad libitum. The urine from each metabolic cage was collected in a tube immersed in ice. The metabolic cages were washed thoroughly with water and sterilized between collections. The urine samples were stored at −80 °C prior to use (Zhang et al. 2019).

Two-way choice tests of interspecific preferences

Forty-three RN females and 41 RT females were used in the two-way choice tests. The behavior experiments were

conducted in a three-chamber testing apparatus constructed from 3 plastic rat cages (37 × 26 × 17 cm). Two cages served as the choice cages and were symmetrically connected to the long side of the neutral cage by two Plexiglas choice tubes (internal diameter, 7 cm; length, 50 cm). Each choice cage was partitioned by a large perforated galvanized iron sheet as a partition. Each tube had a small removable perforated galvanized iron sheet partition as a door placed 10 cm away from the neutral cage to control rat access (Figure 2A).

Experiment 1: Assessment of the attractiveness of RN and RT male rats to female rats. One RN male and RT male were placed in either of the choice cages for 30 min. Then, we placed one female subject of either RN or RT in the neutral cage for 10 min of acclimation and opened the door to allow the female subject to freely respond to the males.

Experiment 2: Assessment of the attractiveness of urinary volatile compounds of RN and RT male rats to female rats. One RN male and one RT male were placed in either of two choice cages, respectively, for 30 min for them to leave scent substance, especially fresh urine (urine marks), and then were removed. One female subject of either RN or RT was placed in the neutral cage and acclimated in the neutral cage for 10 min. We then opened the door to allow the female rat to freely sniff the urine marks, but could not touch and lick the urine.

Experiment 3: Assessment of the attractiveness of urinary volatile compounds and MUPs of RT and RN male rats to female rats. RN males and RT males were placed in either of two choice cages for 30 min and then removed from the choice cages. One female subject of either RN or RT was placed in the neutral cage and acclimated for 10 min. Then, we opened the door of each tube and removed the partition of each choice cage to allow the female rat to freely sniff and lick the urine marks.

The three-chamber testing apparatus was cleaned thoroughly with 75% ethanol and water between trials. We recorded the percentage time (the time each female spent in a choice cage) for 30 min immediately after the focal rat initially entered either of the choice arms (Guo et al. 2017).

Recordings and analysis of USVs

We recorded and analyzed the USVs of 11 RN males and 10 RT males in a mate attraction context (male and female individuals were placed on either side of the box and separated by a grid plate). The USVs were recorded with an Avisoft Bioacoustics USG 116 (e) detector (Avisoft Bioacoustics, Berlin) equipped with an Avisoft FG series microphone connected by a 2-m cable in a soundproofed chamber (3 × 2.3 × 2.5 m). The USVs were recorded when a male encountered a female in a recording cage (50 × 35 × 20 cm) for 30 min. All the rats underwent heterosexual encounters prior to the experiment. In these two subspecies, both sexes emit USVs during mating (McGinnis and Vakulenko 2003). The USVs of RN males and RT males were used to assess the divergence between the two related species. The parameters of USVs were analyzed with Avisoft-SASLab Pro (Avisoft, Germany) and Sound Analysis Pro 2011 software (v 1.04). The spectrograms were generated with a fast Fourier transform length of 1024 points and 87.5% frequency overlap with a Hamming window. The measurements of the duration, peak frequency, bandwidth, and pulse rate provided a quantitative description of USVs. The parameters of USVs produced in a courtship context were measured in each syllable as a unit of sound separated by a silent period before another sound. Trills referred to repeated high-frequency calls with dense peaks recorded in each band of the USV spectrum and represented fast sin wave-like oscillations of the call frequency (Burgdorf et al. 2008; Brudzynski 2015).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of MUPs

The relative abundances of MUPs of 6 RN males and 6 RT males were qualified by SDS–PAGE using the Mini P-4 system (Cavoy, China). Eight microliters of each urine sample were mixed with 8 μL of ddH_2O and 4 μL of 5 × SDS–PAGE loading buffer (Solarbio, China). The mixed protein samples were heated at 100 °C for 5 min. Eight microliters of each mixed urine sample and 6 μL of protein molecular weight marker (low) (TAKARA, Japan) were fractionated on 15% SDS–PAGE gels at a constant voltage of 90 V for 3 h. The protein gels were stained with Coomassie brilliant blue (Sigma-Aldrich) dye for 1 h and then imaged with a ChemiDoc MP system (Bio-Rad, USA). The relative abundances of MUPs were quantified with ImageJ software (National Institutes of Health, USA). One sample was selected as a standard control and fractionated on each gel to correct for bias between runs and to normalize the relative abundances of other samples (Guo et al. 2019).

Isoelectric focusing electrophoresis (IEF) of MUPs

IEF analysis was used to separate proteins based on the isoelectric point of MUPs with Mini Cell equipment (Bio-Rad, USA). The samples of urine of 6 RN males and 6 RT males were used for IEF. The polyacrylamide gel included sterile water (2.75 mL), monomer concentrate (25% T, 3% C, 1 mL), 25% glycerol (w/v, 1 mL), ampholyte (250 μL) with 3–10 PH, 10% ammonium persulfate (w/v, 7.5 μL), 0.1% FMN (w/v, 25 μL), and TEMED (1.5 μL). The desalted and precipitated protein solutions from 130 μL of urine were obtained by desalting columns (ThermoFisher, USA), vacuum freeze drying and dissolution with 10 μL of ddH_2O. One microliter of protein solution and 2 μL of markers (SERVA, Germany) with PI (3–10) was applied to the gel and allowed to diffuse for 5 min. The gel was run under the conditions of 100 V for 15 min and 450 V for 1 h. Targeted bands were imaged by the ChemiDoc MP instrument (Bio-Rad, USA) followed by fixing, staining, and decolouration (Guo et al. 2019).

Data analysis

Kolmogorov–Smirnov tests were used to examine the distribution of raw data, and parametric tests were used for normally distributed data. Independent-sample *t*-tests were used to compare the duration, the peak frequency, the bandwidth, and the abundances of MUPs between RN males and RT males. Independent-sample *t*-tests were conducted using SPSS (version 18.0). Hist was used to examine the distribution of raw data, which led to choose a negative binomial generalized linear model (GLM-NB) with R package (MASS) to test for the investigation time and differences in the pulse rate between RN males and RT males. GLM was performed

with R software (version 4.1.2). The significance threshold was set to $P < 0.05$.

Results

Two-way choice test

Rattus tanezumi (left) and *R. norvegicus* (right) were showed in Figure 1. In Experiment 1, RN females (GLM-NB, $z = 49.210$, $P < 0.010$, $N = 15$) (Figure 2B) and RT females (GLM-NB, $z = 2.154$, $P = 0.031$, $N = 15$) (Figure 2C) approached conspecific males at a higher proportion than the heterospecific males.

In Experiment 2, RN females had no preference for volatile signals produced by RN males or RT males (GLM-NB, $z = 0.718$, $P = 0.472$, $N = 14$) (Figure 2D) and neither did RT females (GLM-NB, $z = 0.921$, $P = 0.357$, $N = 13$) (Figure 2E).

In Experiment 3, RN females had no preference for scent signals of RN males or RT males (GLM-NB, $z = 0.080$, $P = 0.937$, $N = 14$) (Figure 2F). RT females significantly preferred the scent signals of RT males (GLM-NB, $z = 4.851$, $P < 0.010$, $N = 13$) (Figure 2G).

Ultrasonic vocalizations

The characteristics of the USVs emitted by male rats were significantly different between these two rat species (Figure 3A,B). As compared with the USVs in RT males, those in RN males had a longer duration ($t = 1.890$, $P = 0.078$, $N = 11$ for RN males, $N = 10$ for RT males) (Figure 3C), a lower peak frequency ($t = 5.131$, $P < 0.010$, $N = 11$ for RN males, $N = 10$ for RT males) (Figure 3D), a narrower bandwidth ($t = 2.518$, $P = 0.021$, $N = 11$ for RN males, $N = 10$ for RT males) (Figure 3E), and a lower pulse rate (GLM-NB, $z = 3.837$, $P < 0.010$, $N = 11$ for RN males, $N = 10$ for RT males) (Figure 3F). In addition, each band seems to represent a trill with dense peaks recorded and reflected high-frequency calls in RT male rats (Figure 3B).

MUPs

The SDS–PAGE results revealed that RN males had significantly higher MUP levels than *RT* males ($t = 4.485$, $P = 0.001$, $N = 6$) (Figure 4A, B). IEF analysis revealed that the protein bands of total MUPs were more numerous in the urine of RN males than in that of RT males (Figure 4C).

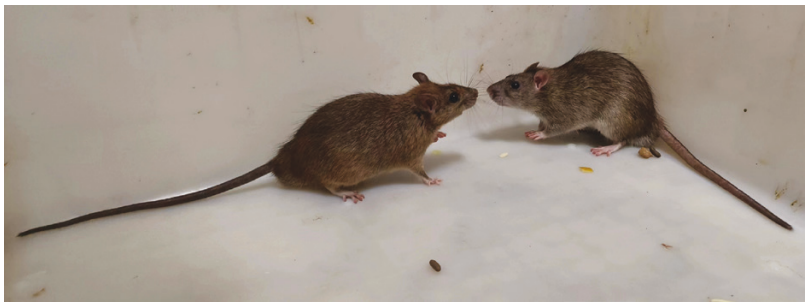

Figure 1. RT (left) and RN (right).

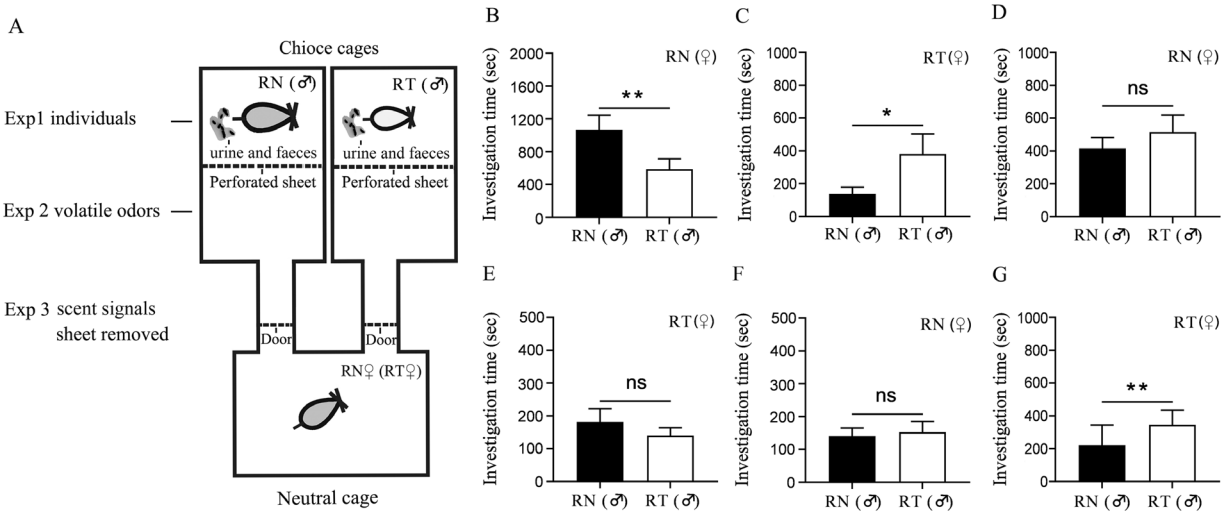

Figure 2. Schematic diagram of the 2-way choice device (A). Investigation time (mean ± *SE*, sec) of RN females or RT females spend for RN males and RT males ($N = 15$ for each species) in Experiment 1 (B and C), for volatile signals (the feces and urine left behind were inaccessible) from RN males and RT males ($N = 14$ RN males, $N = 13$ RT males) in Experiment 2 (D and E), and for volatile and nonvolatile signals (the feces and urine left behind were accessible) from RN males and RT males ($N = 14$ RN males, $N = 13$ RT males) in Experiment 3 (F and G) (*$P < 0.05$, **$P < 0.01$).

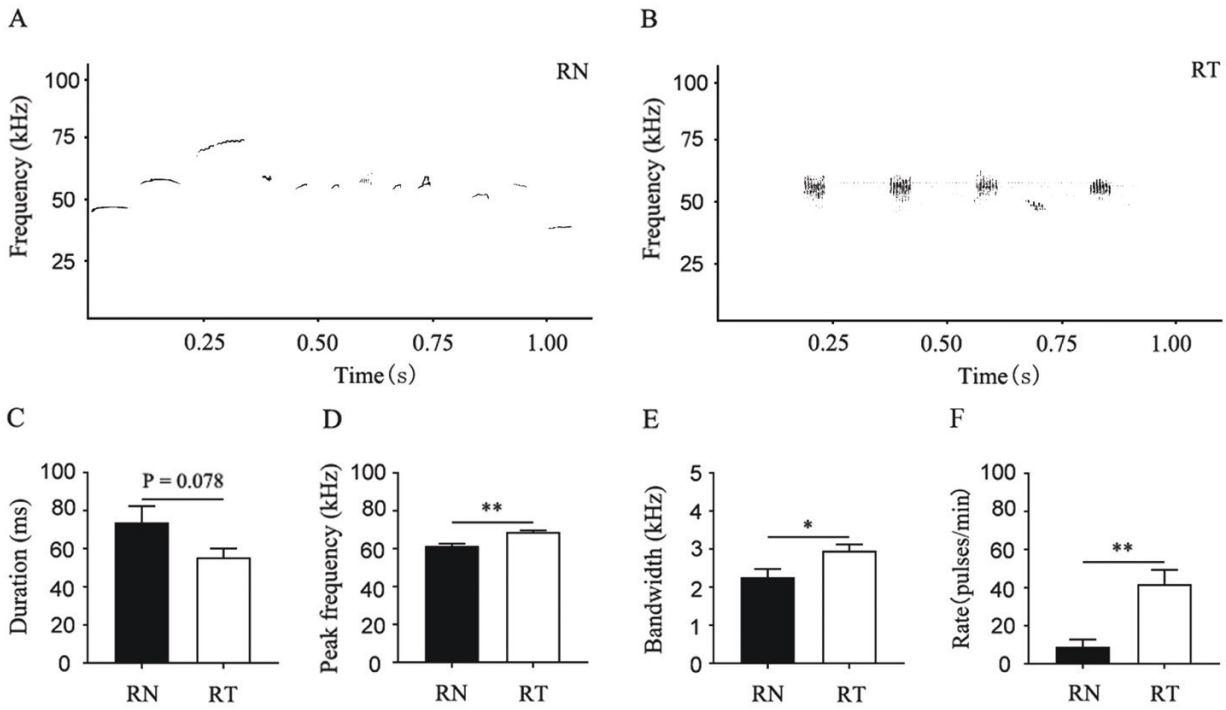

Figure 3. The representative USVs spectrograms of RN males (A) and RT males (B) in a mate attraction context. Representative spectrograms were generated with a fast Fourier transform length of 1024 points and 87.5% frequency overlap with a Hamming window. The difference in USV duration (C), peak frequency (D), bandwidth (E), and pulse rate (F) between RN males and RT males (mean ± *SE*, $N = 11$ for RN males, $N = 10$ for RT males, $^{*}P < 0.05$, $^{**}P < 0.01$).

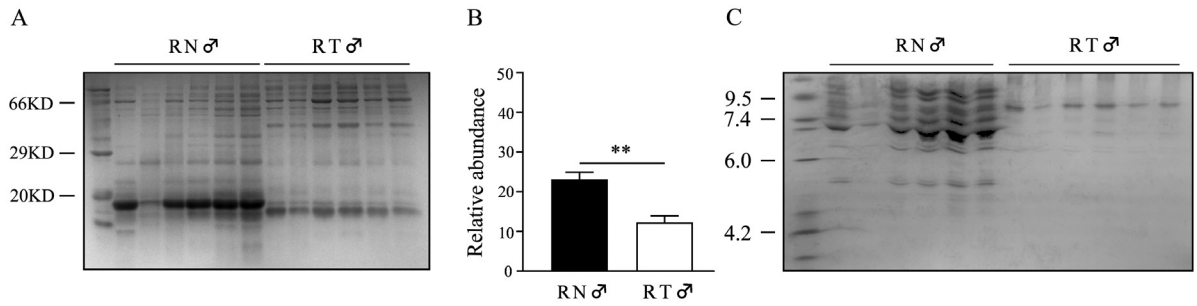

Figure 4. Comparison of MUPs between RN males and RT males. (A) SDS–PAGE image of rat urine samples. (B) Relative abundance of MUPs in rats quantified by SDS–PAGE analysis ($N = 6$, $^{**}P < 0.01$). (C) IEF was used to resolve MUPs in the PI fractions (3.0–10.0). Desalted urine was subsequently focused, stained, and decolored. The overall pattern of MUPs bands significantly differed between RN males and RT males.

Discussion

Our data suggested that females have a conspecific preference only when they can assess a certain combination of signals and cues but not when presented in isolation in these two coexisting rat species as expected. Interspecific discrimination is important for the formation of prozygotic barriers of hybridization to maintain species integrity and interspecies boundaries (Svensson et al. 2007). Coexisting closely related species often evolve strong species discrimination due to the reinforcement of mate preference and character displacement of signals (Higgie et al. 2000; Svensson et al. 2007; Pfennig and Pfennig 2009; Stuart and Losos 2013). Here, the differentiation of USVs and chemical signals between RN and RT male rat species might contribute to species discrimination by females.

More urinary volatile compounds were detected in RNH males than in RT males. The most abundant volatile in RNH males was 2-heptanone, whereas ethyl phenol was the most abundant volatile in RT males. This matches previously published results (Guo 2016). However, volatile cues alone did not significantly influence association preferences of females of either species under the current experimental paradigm (Experiment 2), implying that volatile cues alone might not be enough for interspecific recognition. However, RT females significantly preferred the scent signals of males of the same species to those of opponent species when accessing the deposited urine (Experiment 3), implying contact MUP signals alone (for RT females), or together with the volatiles (both species) are important for species recognition. Both RN females and RT females showed a behavioral preference for conspecific males to heterospecific males when the male demonstrators were separated from choosy females by a net, implying that the USVs alone or together with volatile signals might be the reliable species recognition signals for these two rat species (Experiment 1). The differences in USVs and scent

signals might be related to phylogenetic divergences between these two rat species (Barbosa et al. 2006). In addition, the asymmetry in olfaction-mediated mate preferences observed in Experiment 3 is similar to those observed in swordtails and grasshoppers, rodents and other species, which might be caused by natural selection, sexual selection, or even drift over evolutionary time (Ryan and Wagner 1987; Hochkirch and Lemke 2011; Shurtliff et al. 2013; Cerveira et al. 2019).

As the most common modality of animal communication, chemical communication can convey accurate information about species, sex, and reproductive status for a viable mating in almost all mammal species (Johansson and Jones 2007; Wyatt 2014). Urine-borne volatile pheromones and MUPs of males have been demonstrated to determine the sexual attractiveness of males to female rats as pheromones and can also be detected by prey and predators as allomones (Papes et al. 2010; Zhang and Zhang 2014; Zhang et al. 2016, 2019; Guo et al. 2017). In house mice (*Mus musculus*), the composition and content of MUPs vary among geographic populations and subspecies and could regulate the recognition between subspecies and partial behavioral reproductive isolation (Hurst et al. 2017). In moths and fruit flies, it was found that small changes in ratios or types of isomers of pheromones could create a barrier that leads to reproductive isolation between closely related species (Löfstedt 1993; Bengtsson and Löfstedt 2007; Wyatt 2014). In the current work, RN males and RT males expressed species-specific predominant MUPs with different molecular weights (18–20 kDa) in the urine (Guo 2016; Wang et al. 2022) (unpublished data). Which MUPs, alone or in combination with urinary volatile signals, served as a reliable signal for the species recognition by female RT rats needed to be further studied.

USVs play diverse roles such as sexual arousal and species recognition in rodents. In mice, it has been exemplified that pheromones may elicit initial responses from conspecifics of the opposite sex and subsequent emission of USVs and behavioral interaction (Demir et al. 2020). In rats, closely related species often exhibit completely different USV characteristics revealed by quantitative analysis (Gerhardt 2001; Boughman 2002; Chen et al. 2017). The current results showed that the USVs of RN males and RT males had different duration, peak frequency, bandwidth, and pulse rate, implying that the USVs were significantly differentiated between RN males and RT males and might contribute to species discrimination. In addition, USVs were sexually distinct in RT rats but not in RN rats, implying USVs might contribute to sex recognition and sexual attractiveness in RT rats (Supplementary Figure S1).

In conclusion, these two coexisting rat species showed expected interspecific recognition in females and emitted species-specific chemical signals and ultrasonic signals in males. These signals might work alone or synergistically to mediate species recognition and mate choice of females in these rat species. In the genus *Rattus*, the rat species are so similar in morphology that we can hardly tell the rat species with the naked eye, implying that the visual signals between rat species might be indistinguishable (Rowe et al. 2011). Therefore, the coexisting rat species might mainly rely on olfactory communication and auditory communication to recognize each other and regulate interspecies relationship (Rowe et al. 2011; Guo et al. 2016, 2017; Chen et al. 2017). In order to better understand species recognition in nature, it is important to examine the evolution of interspecific signals or their role and determine whether sympatry contributes to further divergence in species signals and leads to character displacement by studying different populations in the future (Higgie et al. 2000; Pfennig and Pfennig 2009; Stuart and Losos 2013).

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Author Contributions

J.X.Z. and Y.H.Z. conceived the study. W.C.W. and Z.M.L. performed the MUP analysis. Y.C. conducted the ultrasonic vocalization analysis; J.H.Z., Y.H.Z., and Z.M.L. conducted the two-way choice tests and took care of the rats. W.C.W., Y.H.Z., and J.X.Z. wrote the manuscript. All the authors read and approved the final manuscript.

Conflict of interests

The authors declare that they have no conflicts of interest related to this work.

Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

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