Call divergence in three sympatric *Rattus* species

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To reduce errors in species recognition and the probability of interbreeding that lowers fitness, individuals within sympatric zones shift the signals to differentiate from those of other species. In the present study, the differences of the acoustic features of ultrasounds (courtship calls during heterosexual encounters) and audible calls (distress calls during tail-clamp stress) are compared among three sympatric *Rattus* species (*Rattus andamanensis*, *R. norvegicus*, and *R. losea*). Results showed that the three species have significantly different call parameters, including call duration, peak frequency, bandwidth, pitch, goodness of pitch, frequency modulation, and Wiener entropy. This study provides quantitative evidence for character displacement in the acoustic signals of closely related sympatric *Rattus* species. Results indicate that the divergence of acoustic signal has arrived at the quite meticulous degree of evolution. Therefore, the acoustic signal trait is likely involved in the evolution of species diversity in rodents. © 2017 Acoustical Society of America.

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I. INTRODUCTION

Character displacement occurs when more than one species inhabits the same area and the morphological, ecological, or behavioral traits of these species diverge (Dayan and Simberloff, 2005; Grant and Grant, 2006). For example, individuals shift signals that are used in communication between mates to differentiate from those of other species within sympatric zones (Miller, 1982). This phenomenon facilitates species recognition and reduces the probability of interbreeding in which hybrids have lower fitness (Shy, 1984). Consequently, character displacement has important implications for understanding species coexistence in natural communities and the processes that lead to speciation and diversification (Kirschel *et al.*, 2009).

Individuals that use the acoustic scene should be able to differentiate between their sound sources and those of other species to perform the activities that are necessary for survival and reproduction (Dent *et al.*, 2016). There are some lines of evidence for call divergence in frogs (Hobel and Gerhardt, 2003; Hoskin *et al.*, 2005), crickets (Jang and Gerhardt, 2006), and songbirds (Shy, 1984; Balakrishnan and Sorenson, 2006), as well as plumage divergence in avian species (Saetre *et al.*, 1997). However, few studies have investigated character displacement in the vocalization calls of rodents (Dempster and Perrin, 1994; Kapusta and Sales, 2009), especially in *Rattus* species whose acoustic signals contain repeated syllables and complex structure like mice (Portfors, 2007).

Myomorphs (murid rodents) produce ultrasonic vocalizations (USVs) in a range of social situations, such as reproductive activity, rough and tumble play, and exposure to aversive stimuli (Sewell, 1968; Nyby and Whitney, 1978; Wöhr and Schwarting, 2013). Conventional studies have suggested that

both male and female rats emit courtship USVs (50-kHz calls) during heterosexual encounters to enhance their sexual responsiveness (Barfield *et al.*, 1979; White and Barfield, 1987, 1990; McGinnis and Vakulenko, 2003). Females prefer to dart toward males who emit more courtship calls (Thomas *et al.*, 1981; Thomas *et al.*, 1982). Females are less receptive when paired with devocalized males (White and Barfield, 1990). More recent studies, however, demonstrated that devocalized rats copulate exactly in the same manner as vocalizing rats (Ågmo and Snoeren, 2015; Chu *et al.*, 2017), suggesting that the functions of USVs during copulation behavior are overestimated in rats. The differences of these results may be attributed to the oestrus stage of females or the experimental condition. Nonetheless, USVs could play a role in signaling motivational state, identity, or mediating their mating behavior during opposite-sex interactions. Moreover, like other animals, rats emit loud audible sounds (pain cry) when subjected to a severe aversive stimulus (Simonov, 1983). Given the importance of interspecific differences in acoustic signals in species evolution, research data are sorely in need to fill the large gaps in *Rattus*. We report a case of character displacement in *Rattus andamanensis*, *R. norvegicus*, and *R. losea* that coexist in the croplands of south China (Aplin *et al.*, 2003; Smith and Xie, 2009). We compared the differences in the acoustic features of courtship USVs and distress calls during tail-clamp stress among these three species. We predict that call divergence exists among these species, and that their acoustic structures will exhibit interspecific discrimination.

II. METHODS

A. Subjects

Adult *R. andamanensis*, *R. norvegicus*, and *R. losea* were all collected from a single farmland area in Zengcheng City, Guangdong Province, China (23°18' N,

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113°38' E). The rats were caged and transported by car to Guangdong Institute of Applied Biological Resources, Guangzhou City, Guangdong Province, People's Republic of China (23°5.7' N, 113°17.4' E), where the experiments were performed. The rats were individually housed in plastic cages (30 × 20 × 16 cm) in a temperature- and humidity-controlled colony room (22–25 °C, 50%–60%). The rats were maintained under a 12/12 dark–light cycle with *ad libitum* access to food (rat pellets produced by Guangdong Medical Laboratory Animal Center) and water for 10 weeks. All procedures were conducted on the basis of the guidelines of the Institutional Animal Care and Use Guangdong Institute of Applied Biological Resources, Guangdong Academy of Sciences.

B. Sound recording and analysis

We recorded the courtship calls of 10 male–female encounters and the distress calls of 10 individuals (5 males and 5 females) from each species. Rat vocalizations were recorded with an Avisoft Bioacoustics USG 116(e) detector (Avisoft Bioacoustics, Berlin) equipped with an Avisoft FG series microphone on a 2-m cable in a soundproofed chamber (3 × 2.3 m and 2.5 m high). The calls were recorded at a sampling rate of 250 kHz. Courtship USVs were recorded when a male encountered a female in the recording cage (50 × 35 × 20 cm) for 30 min. Individuals used in the courtship experiment were sexually experienced. They all underwent heterosexual encounters in pre-experiment. In this case, both sexes of rats emit plenty of ultrasonic vocalizations during mating (Thomas *et al.*, 1981; Thomas and Barfield, 1985; White and Barfield, 1987; White *et al.*, 1993; McGinnis and Vakulenko, 2003). We thus used USVs of both males and females to assess the call divergence among the three species. The rats' audible distress calls were also recorded with tail-clamp method (Engelhardt *et al.*, 1995). Individuals were only used once for this experiment.

Rat vocalization parameters were analyzed with Avisoft-SASLab Pro and Sound Analysis Pro 2011 software (v 1.04). Discriminant analysis of the calls in the three species based on these parameters: duration (onset of sound to end), peak frequency (the frequency that was produced at the maximum amplitude), bandwidth (computed as highest frequency minus lowest frequency at –30 dB), pitch (highest peak in the cepstrum), goodness of pitch (how much of the sound energy is concentrated in the pitch and its harmonics), FM (frequency modulation), and Wiener entropy (a measure of the breadth and evenness of the spectrum of the sound). Duration, peak frequency, and bandwidth were measured with Avisoft-SASLab Pro. Spectrograms were generated with a fast Fourier transform length of 1024 points and 87.5% frequency overlap with a Hamming window. Pitch, goodness of pitch, FM, and Wiener entropy were obtained with Sound Analysis Pro 2011 software (Tchernichovski *et al.*, 2000; Baker, 2011; Tchernichovski, 2012). The measurements of these seven acoustic features provide a quantitative description of rat vocalizations. Rat ultrasonic vocalizations consist of bouts of calls separated into syllables by periods of silence, and each type of courtship call consists of a variable number of syllables uttered in a sequence (Fig. 1). Thus, courtship call parameters were measured from each syllable. In total, we extracted and analyzed 6497 ultrasonic syllables from 2985 courtship calls and 1067 audible calls.

C. Statistical analyses

The mean value per individual was used for statistical analysis. The statistical analyses were conducted using SPSS 16.0 for Windows. The normality and homogeneity of the variances were examined before analysis. Statistical comparisons were conducted by performing one-way analysis of variance (ANOVA) followed by Tukey's test. Non-parametric statistical tests were used for data that violated the homogeneity of variance assumption. We used the

FIG. 1. (Color online) The characteristics of courtship calls during heterosexual encounters of *Rattus andamanensis*, *R. norvegicus*, and *R. losea*. (a) *R. andamanensis*; (b) *R. norvegicus*; (c) *R. losea*.

TABLE I. Physical characteristics of audible calls during tail-clamp of three species of *Rattus*. Values given are means $\pm$ SE. Means with the different letters (case insensitivity) differ significantly: A, B: $P < 0.05$; a, b: $P < 0.01$.

Species	<i>R. andamanensis</i>	<i>R. norvegicus</i>	<i>R. losea</i>	Statistic
Duration (s)	689.15 $\pm$ 71.22 ^a	312.12 $\pm$ 45.02 ^b	226.58 $\pm$ 30.15 ^b	$F_{2,27} = 24.547, P < 0.01$
Peak frequency (kHz)	8.72 $\pm$ 0.12 ^a	8.49 $\pm$ 0.17 ^a	5.70 $\pm$ 0.20 ^b	$F_{2,27} = 98.182, P < 0.01$
Bandwidth (kHz)	0.32 $\pm$ 0.04 ^B	0.48 $\pm$ 0.06 ^{A,B}	0.77 $\pm$ 0.16 ^A	$\chi^2_{2,27} = 7.228, P < 0.05$
Pitch (kHz)	3.87 $\pm$ 0.15 ^a	2.64 $\pm$ 0.14 ^b	3.20 $\pm$ 0.44 ^a	$\chi^2_{2,27} = 9.672, P < 0.01$
Goodness of pitch	68.28 $\pm$ 0.87 ^a	49.76 $\pm$ 2.81 ^b	73.61 $\pm$ 2.48 ^a	$F_{2,27} = 31.811, P < 0.01$
FM (Hz)	46.04 $\pm$ 0.44 ^a	33.85 $\pm$ 2.35 ^b	46.77 $\pm$ 0.95 ^a	$\chi^2_{2,27} = 18.243, P < 0.01$
Entropy	-2.74 $\pm$ 0.09 ^B	-3.00 $\pm$ 0.19 ^b	-2.08 $\pm$ 0.25 ^{a, A}	$F_{2,27} = 6.340, P < 0.01$

Kruskal-Wallis one-way analysis of variance followed by the Mann-Whitney U test with Bonferroni's correction when the Kruskal-Wallis test was significant. The statistical significance was set at $P < 0.05$. The measured parameters were expressed as the mean $\pm$ SE.

III. RESULTS

In sympatry, all three rat species emitted audible calls during tail-clamp stress and ultrasounds during heterosexual encounters. No significant differences in audible calls exist between sexes in all three species (Independent-sample t -test: all $P > 0.05$). One-way analysis of variance (ANOVA) analysis showed that the parameters of audible and ultrasonic calls were significantly different among the three rat species, including duration (audible sound: $F_{2,27} = 24.547, P < 0.01$; USV: $\chi^2_{2,27} = 10.447, P < 0.01$), peak frequency (audible sound: $F_{2,27} = 98.182, P < 0.01$; USV: $F_{2,27} = 6.424, P < 0.01$), bandwidth (audible sound: $\chi^2_{2,27} = 7.228, P < 0.05$; USV: $F_{2,27} = 25.620, P < 0.01$), pitch (audible sound: $\chi^2_{2,27} = 9.672, P < 0.01$; USV: $F_{2,27} = 144.147, P < 0.01$), goodness of pitch (audible sound: $F_{2,27} = 31.811, P < 0.01$; USV: $\chi^2_{2,27} = 20.015, P < 0.01$), FM (audible sound: $\chi^2_{2,27} = 18.243, P < 0.01$; USV: $\chi^2_{2,27} = 19.726, P < 0.01$), and entropy (audible sound: $F_{2,27} = 6.340, P < 0.01$; USV: $F_{2,27} = 41.123, P < 0.01$).

A. Audible calls during tail-clamp

The audible calls of *R. andamanensis* were longer in duration than those of the other two species (Tukey: $P < 0.01$). Moreover, the bandwidths of *R. andamanensis* were narrower than those of *R. losea* ($Z = -2.422, P < 0.05$) and their pitches were higher than those of *R. norvegicus* ($Z = -3.704, P < 0.01$). The calls of *R. losea* show lower peak frequency (Tukey: $P < 0.01$) but higher entropy

(Tukey: $P < 0.05$) than those of the other two species. Additionally, *R. norvegicus* emitted audible calls with lower goodness of pitch (Tukey: $P < 0.01$) and FM (*R. andamanensis*: $Z = -3.704, P < 0.01$; *R. losea*: $Z = -3.628, P < 0.01$) than either *R. andamanensis* or *R. losea* (Table I).

B. Ultrasonic vocalizations during heterosexual encounters

The courtship USVs of *R. andamanensis* were longer (*R. norvegicus*: $Z = -2.969, P < 0.01$; *R. losea*: $Z = -2.522, P < 0.01$) and narrower (Tukey: $P < 0.01$), and their pitches were higher than those of the other two species (Tukey: $P < 0.01$). The goodness of pitch (*R. andamanensis*: $Z = -3.78, P < 0.01$; *R. norvegicus*: $Z = -3.78, P < 0.01$) and FM (*R. andamanensis*: $Z = -3.78, P < 0.01$; *R. norvegicus*: $Z = -3.78, P < 0.01$) of USVs of *R. losea* were both lower than those of the other two species. *R. norvegicus* emitted USVs with a lower peak frequency than the other two species (Tukey: $P < 0.01$). Additionally, the entropies of USVs were different among the three species (Tukey: $P < 0.01$). The highest entropy value was observed for *R. norvegicus* and the lowest for *R. andamanensis* (Table II).

IV. DISCUSSION

The current study compared audible calls and USVs among three *Rattus* species. Our results confirmed character displacement in the vocalization calls of these species. We quantitatively examined the differences in vocalizations under tail-clamp stress and over the course of courtship interaction. Results showed that the three species of wild rats have significantly different call parameters, including call duration, peak frequency, bandwidth, pitch, goodness of pitch, frequency modulation, and Wiener entropy. The

TABLE II. Physical characteristics of ultrasonic calls during conspecific interactions between male-female pairs of three species of *Rattus*. Values given are means $\pm$ SE. Means with the different letters (case insensitivity) differ significantly: a, b, c: $P < 0.01$.

Species	<i>R. andamanensis</i>	<i>R. norvegicus</i>	<i>R. losea</i>	Statistic
Duration (s)	228.06 $\pm$ 54.88 ^a	56.31 $\pm$ 2.38 ^b	52.56 $\pm$ 2.85 ^b	$\chi^2_{2,27} = 10.447, P < 0.01$
Peak frequency (kHz)	65.30 $\pm$ 2.46 ^a	54.39 $\pm$ 2.31 ^b	63.47 $\pm$ 1.82 ^a	$F_{2,27} = 6.424, P < 0.01$
Bandwidth (kHz)	0.91 $\pm$ 0.16 ^b	2.16 $\pm$ 0.14 ^a	2.32 $\pm$ 0.13 ^a	$F_{2,27} = 25.620, P < 0.01$
Pitch (kHz)	60.00 $\pm$ 1.43 ^a	21.94 $\pm$ 1.56 ^b	25.59 $\pm$ 2.17 ^b	$F_{2,27} = 144.147, P < 0.01$
Goodness of pitch	65.46 $\pm$ 0.46 ^a	66.61 $\pm$ 1.85 ^a	46.71 $\pm$ 1.47 ^b	$\chi^2_{2,27} = 20.015, P < 0.01$
FM (Hz)	42.01 $\pm$ 0.32 ^a	42.15 $\pm$ 1.27 ^a	26.20 $\pm$ 1.26 ^b	$\chi^2_{2,27} = 19.726, P < 0.01$
Entropy	-2.42 $\pm$ 0.09 ^c	-1.19 $\pm$ 0.07 ^a	-1.97 $\pm$ 0.12 ^b	$F_{2,27} = 41.123, P < 0.01$

species differences in acoustic signals possibly occurred due to physical differences, such as thoracic or laryngeal size (Scattoni *et al.*, 2008; Tabler *et al.*, 2017). The production of USVs is closely related to the expiration and laryngeal braking (Roberts, 1972; Mortola, 1987; Tabler *et al.*, 2017). The differences in anatomical structure (laryngeal or tracheal apparatuses) may account for the species differences in acoustic signals (Reznik, 1990; Hashimoto *et al.*, 2004). For example, larger instruments often produce lower frequencies, which have been demonstrated in many species (Ryan *et al.*, 1992; Fitch, 1997; Hoskin *et al.*, 2009). In this study, the difference in lung volume originating from body size might be connected with the specific characteristics of USVs, as *R. norvegicus* with the larger lung volume and body size emitted the USVs that were lower in frequency. Another obvious consideration is that each species has more auditory experience with conspecific, rather than heterospecific vocalizations (Dooling *et al.*, 1992). Previous studies have shown that auditory cortical circuits of rats can be shaped by experience and learning (Dahmen and King, 2007). However, cross-fostering experiments revealed that acoustic communication in mice is innate and not learned (Kikusui *et al.*, 2011). Then cross-fostering experiments would be better at revealing whether and how experience or learning influences acoustic communication in rats. Furthermore, their different microhabitats or original habitats may enable sympatric species to create their own acoustic traits (de Freitas *et al.*, 2015).

Previous studies have shown that rats display avoidance reactions to the pain cry of another individual (Simonov, 1983), which suggests that a stress call under discomfort or threatened condition contains an alarm function for another individual. In some sympatric mammals and birds, alarm calls have converged among species with similar predators. These convergent alarm calls are remarkably similar to each other (Roux *et al.*, 2002). However, alarm calls are often species-specific and can, therefore, be used to distinguish between closely related rodent species, although they are not as effective in conspecific species-recognition as mate calls (Roux *et al.*, 2002). Thus, pain calls that are remarkably different among the three species may be involved in species recognition, which is a common function of many vocal signals. There is considerable evidence that animals, including humans, perceive species-specific acoustic communication signals differently than acoustic signals from other species (Moody *et al.*, 1990).

Multi-species communities are widespread in the natural world. Consequently, distinguishing and recognizing mates between conspecific and heterospecific individuals are important for these communities. Natural selection favours traits that increase assortative mating, thereby reducing the frequency of maladaptive hybridization (Dobzhansky, 1940; Haavie *et al.*, 2004). One important approach to assortative mating is reinforcement, which will cause signals used in mate recognition to diverge and reinforce the pre-zygotic barrier, eventually completing speciation in sympatry (Saetre *et al.*, 1997). Our resultant evidence is strongly indicative that the mate calls of sympatric *Rattus* are species-specific. Given the large variances in call structures, *Rattus*

species may distinguish between conspecific and heterospecific individuals based on their mating calls, which has been demonstrated in mice (Musolf *et al.*, 2015). We could not deny that the differences found maybe attributed to the differences between sexes, because we were not able to distinguish the courtship calls between males and females. However, the structural characteristics (mainly in frequency and duration) and the number of USVs show no difference between sexes in rats (Thomas and Barfield, 1985). This means that males and females contribute equally to total vocal sound and have the same effect on the results of the acoustic characteristics in each species.

Rats emit 50-kHz calls in different contexts including rough-and-tumble play (Knutson *et al.*, 1998; Brunelli *et al.*, 2006), mating (Sales, 1972; McIntosh *et al.*, 1978; Burgdorf *et al.*, 2008; Seffer *et al.*, 2012), and social contact (Brudzynski and Pniak, 2002; Schwarting *et al.*, 2007). Burgdorf *et al.* found that rats self-administered playback of frequency modulated 50-kHz trill calls (Burgdorf *et al.*, 2008). Wöhr and Schwarting also demonstrate that 50-kHz signals can induce rat approach behavior (Wöhr and Schwarting, 2007, 2009). However, recent evidence shows that the courtship USVs do not have any consistent incentive value for the sexually experienced male or female rats (Snoeren and Ågmo, 2013, 2014). Although the differences of rat 50-kHz calls in different situations are still unclear, we may reasonably conclude that rat 50-kHz calls are a social thing rather than a sexual thing. Therefore, rat courtship USVs probably do not function as a cue for sexual attraction like they do for mice (Hammerschmidt *et al.*, 2009; Shepard and Liu, 2011; Neunuebel *et al.*, 2015; Chen *et al.*, 2017), and they may just perform the function of establishing and maintaining close social contact. Moreover, rat courtship USVs may indicate merely a positive affective state. Given the species-specific characters of the calls in sympatric *Rattus*, it should also be considered that the recognition function could be involved in their vocal behavior. Rats are highly social animals and vocal communication is a regular feature of their social interactions (Brudzynski, 2009). Obviously, communication should establish on mutual recognition.

Several reports have shown that call divergence exists in sympatric voles (Kapusta and Sales, 2009) and gerbils (Dempster and Perrin, 1994). However, these studies mainly focus on the duration and frequency of sound features, which cannot comprehensively measure acoustic structures. In the present study, we quantitatively examined various aspects of call structures, such as goodness of pitch and entropy, in addition to duration and frequency. Goodness of pitch is an estimate of harmonic pitch periodicity. Wiener entropy is a measure of the width and uniformity of the power spectrum. These parameters are no less important than duration and frequency in describing acoustic signal. In conclusion, the present study first provided evidence for character displacement in the acoustic signal of *Rattus*. Furthermore, our results revealed that the vocal traits of *Rattus* were likely exposed to strong selection pressure in evolutionary terms. Although acoustic traits alone cannot be used as taxonomic indices, revealing the differences and divergences of these

characteristics can contribute to phylogenetic analysis and construction in rats.

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