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Odorants Differentiate Australian *Rattus* with Increased Complexity in Sympatry

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Abstract. Odorant cues play a critical role in premating isolation among many species. In mammals, they have been most well-studied in rodents, but only in a handful of species. The genus *Rattus* is one of the most species-rich genera of mammals, with a natural distribution from Asia to Australia and a nearly global distribution for a few species that spread through human commensalism. More than one-third of *Rattus* species are the result of a recent and rapid radiation on continental Australia (Sahul) centred on the island of New Guinea. The two most widespread species resulting from this radiation, *Rattus fuscipes* and *Rattus leucopus,* occur sympatrically in the Wet Tropics region of Queensland, Australia. Despite their recent divergence, morphological similarity, and ability to produce fertile offspring in captivity, hybrids of the two species have not been reported in the wild, suggesting that premating isolation mechanisms maintain the species' boundaries. Odorant cues are a plausible mechanism that these species could use to identify mates of the same species, but the chemical composition of their odours has not been characterized. With allozyme data from 166 specimens of the two species we confirmed the absence of gene flow between the species in sympatry. From chemical analysis of preputial glands of 32 males from sympatric and allopatric populations of the two species we identified 120 volatile organic compounds of which 80 were reliably quantitated for statistical analysis. Some of these chemicals have been indicated as signalling compounds in other species of mammals, including seven thiazolines. Among them two (2-sec-butylthiazoline and 2-isopropythiazoline) have been previously detected in a rodent, the House Mouse, *Mus musculus*, and are involved in social interactions including attracting females. We demonstrate that *R. fuscipes* and *R. leucopus* are quantitatively and qualitatively distinguishable by the chemical composition of their preputial gland secretions. In comparison to allopatric subspecies, sympatric species contained more unique chemical compounds and a higher abundance of compounds overall, suggesting that sympatric populations have more complex and concentrated odours. Together these results indicate that odorant chemistry has evolved rapidly in these two species, with substantial differences among species and subspecies, especially in sympatry. Ultimately, the rapid evolution of chemical signals involved in mate recognition may help to explain the exceptional diversity of species in the genus *Rattus.*

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Introduction

A gifted mammalogist, like Dr Ken Aplin, can identify similar and closely related but reproductively isolated species using only morphology. Defining reproductively isolated species in this way involves arduous quantitative and qualitative examination of many specimens distributed across large geographic areas and collected over many years (e.g., Patton *et al*., 2008; Aplin *et al*., 2015). While mammalian taxonomy is increasingly integrative, incorporating morphology, genetics, acoustics, environment, and other variables (e.g., Dayrat, 2005; Padial *et al*., 2010; Schlick-Steiner *et al*., 2010; Phuong *et al*., 2014; Shekelle *et al*., 2017), the vast majority of species have been described with only morphological data (Wilson & Reeder, 2005; Burgin *et al*., 2018). While mammalian species descriptions rely heavily on internal morphological characters, mammalian species themselves rely on other visible, auditory, or chemosensory characters to identify mates of the same species (Panhuis *et al*., 2001; Smadja & Butlin, 2009).

Chemical communication is the oldest and most widespread form of communication in nature and plays a central role in social interactions such as mate choice, parental care, territoriality, sociality, and species recognition (Burger, 2005; Brennan & Kendrick, 2006; Hull & Domingues, 2007; Ferrero & Liberles, 2009, Steiger *et al*., 2011). For invertebrate species, chemical cues are a primary mechanism for choosing mates of the same species and have been used to delimit species boundaries (Linn & Roelofs, 1995; Blows & Allan, 1998; Higgie *et al*., 2000; Lassance *et al*., 2019). Chemical cues, odorants, also play an essential role in mate choice in many mammalian species, but our knowledge of odorant variation is limited to very few mammalian species. In rodents they have been most completely characterized in the House Mouse, *Mus musculus*, where over 100 compounds have been identified that affect a host of reproductive behaviours and conditions including estrous induction, puberty onset, intermale aggression, and female attraction (Novotny *et al*., 1990, 2003; Zhang *et al*., 2007; Novotny & Soini, 2013). Closely related rodent species often have distinct chemical signatures suggesting that these cues are useful in maintaining species boundaries and the rapid evolution of odorant signatures after speciation (Lane *et al*., 2004; Smadja & Butlin, 2009). For example, closely related species of *Mus* (e.g., *M. musculus* and *M. spicilegus;* Soini *et al*., 2009), subspecies of *M. musculus* (*M. m. musculus* and *M. m. domesticus*; Smadja & Ganem, 2002) and lab-derived strains of *M. musculus* (Zhang *et al*., 2007) are each distinguishable by chemical compounds in their urine. Between *Mus musculus* subspecies, these differences have been linked to premating isolation, especially in sympatry (Smadja & Ganem, 2007; Bimova *et al*., 2009; Hurst *et al*., 2017). The role of chemical cues in differentiating closely related species and their involvement in premating isolation has been studied in a handful of genera from a few families of rodents including Cricetidae (*Graomys,* Theiler & Blanco, 1996; *Mesocricetus*, Johnston & Robinson, 1993; *Microtus*, Welsh *et al*., 1988*; Peromyscus*, Moore, 1965; and *Phodopus,* Soini *et al*., 2005); Spalacidae (*Spalax*, Nevo *et al*., 1976); and Muridae (*Mus*, Kotenkova & Naidenko, 1999; *Mastomys*, Apps *et al*., 1990; *Otomys*, Pillay *et al*., 1995; *Rattus*, Kannan *et al*., 1998; and *Rhabdomys*, Pillay *et al*., 2006). However, chemical cues involved in species boundaries have not been examined in the vast majority of rodent species. Given that these cues are species-specific, our ability to extend these patterns across the diversity of rodents is limited by the paucity of species examined (Brennan & Zufall, 2006).

The most significant sources of odorant cues in rodents are bladder urine and preputial glands. Preputial glands are specialized subdermal exocrine glands that empty directly into the urinary tract, providing many of the odorant signals found in urine (Brown & Williams, 1972; Orsulak & Gawienowski, 1972; Novotny, 2003). Secretions from preputial glands are composed of a large number of volatile compounds immersed in a complex of proteins, especially the major urinary proteins (Brown & Williams, 1972; Novotny, 2003). The preputial glands of rodents have an independent origin from the preputial glands of other mammals, such as those of artiodactyls (Brown & Williams, 1972). Preputial gland anatomy varies widely among rodent species, including absence in some species (Brown & Williams, 1972; Breed *et al*., 2020). In the genus *Rattus* and its closest relatives, the preputial gland is large and prominent (Jackson, 1938; Mallick, 1991; Natynczuk *et al*., 1995). In many species of rodents, including *Rattus,* males have more developed preputial glands than females suggesting a role in sexual selection (Kannan *et al*., 1997). Studies of preputial gland secretions in rodent species show that they are involved in social signalling, including identifying conspecifics and maintaining species boundaries (Bronson & Caroom, 1971; Brown & Williams, 1972; Orsulak & Gawienowski, 1972; Welsh *et al*., 1988; Novotny, 2003; Kamalakkannan *et al*., 2006; Zhang *et al*., 2008a).

With 68 recognized species, *Rattus* is the most species-rich genus of rodent (Burgin *et al*., 2018; Mammal Diversity Database, 2019). The genus has diversified recently and rapidly since its origin in the Pliocene, with a high degree of morphological similarity retained among species (Rowe *et al.,* 2011). More than one-third of *Rattus* species (n = 25) are native to continental Australia (Sahul) and evolved from a single colonization of New Guinea circa 1 million years ago (Rowe *et al*., 2011). From an origin on the Asian continent, *Rattus* colonized Sahul via the island archipelago of Wallacea during the Pleistocene and the subsequent diversification of *Rattus* on Sahul is among the most rapid reported for mammals (Rowe *et al*., 2011, 2019). The two most widespread species of Australian *Rattus*, *R. fuscipes* and *R. leucopus*, are sympatric in mid-elevation rainforests of the Wet Tropics region of Queensland Australia between Cooktown and Townsville (Fig. 1), but allopatric throughout the remainder of their respective ranges. *Rattus fuscipes* is distributed from the Wet Tropics south along the eastern, southern, and southwestern coasts of Australia whereas *R. leucopus* is distributed from the Wet Tropics north into southern New Guinea. Where they are sympatric, they are recognized as the subspecies *R. f. coracius* (*Rfc*) and *R. l. cooktownensis* (*Rlc*), respectively. Both subspecies are separated from their geographically closest subspecies by a gap in their distribution; i.e. *R. f. assimilis* (*Rfa*) from south of MacKay, QLD and *R. l. leucopus* (*Rll*) from north of Coen, QLD (Fig. 1). In sympatry, the two species are notoriously difficult to distinguish based on external morphological characters (Taylor & Calaby, 1988; Lidicker & Laurance, 1991), and laboratory crosses between the species have produced fertile offspring in captivity (Baverstock *et al*., 1983). However, the species can be

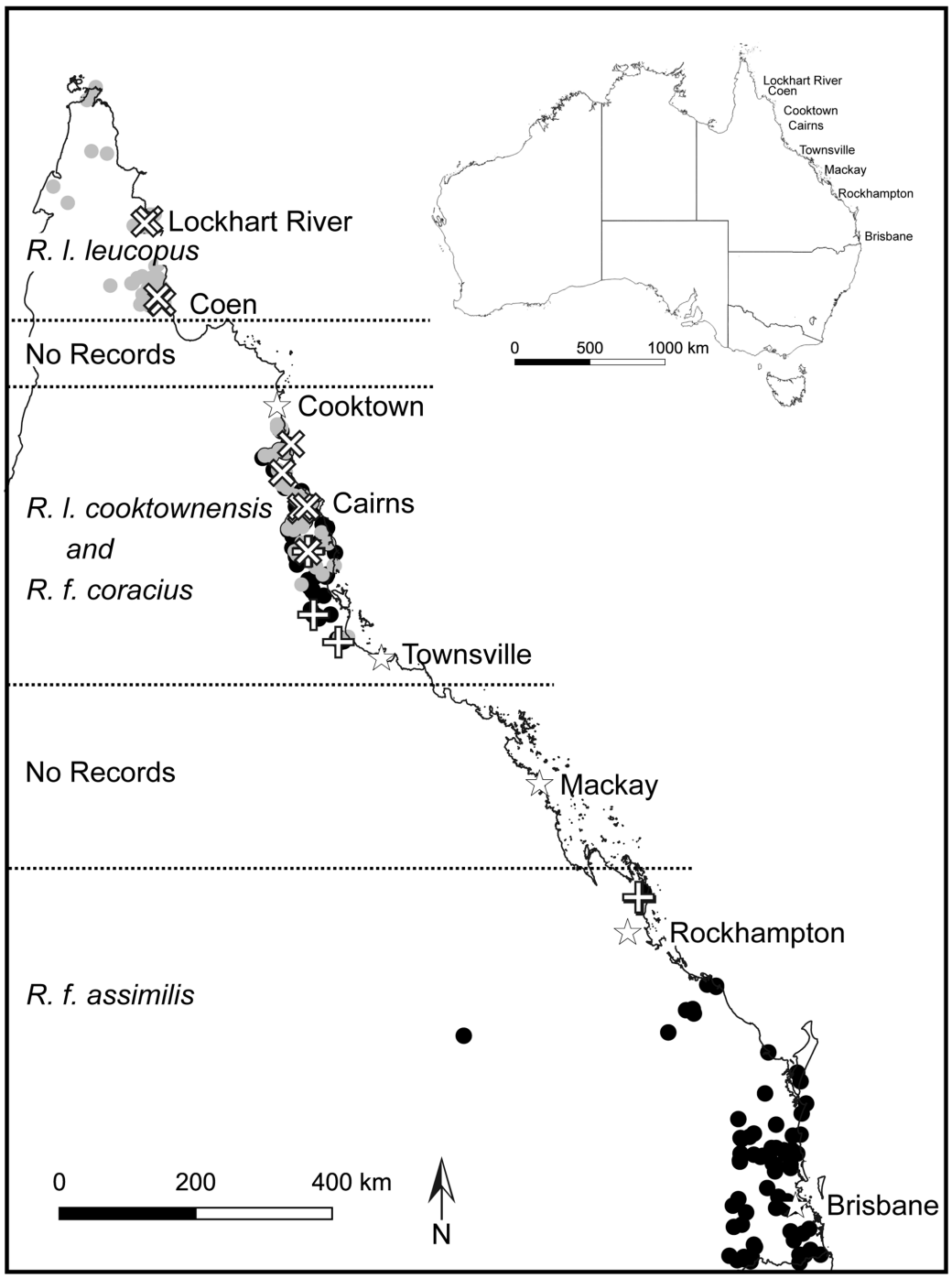

Figure 1. Map of sample localities across Queensland with select cities and towns indicated with stars. Preputial gland sample localities are indicated by open plus symbols (*Rattus fuscipes*) or open crosses (*R. leucopus*). Allozyme samples (not shown) were obtained from the same or nearby localities that are indistinguishable at this scale. Circles show the geographic distribution of all specimen records of *R. fuscipes* (black) and *R. leucopus* (grey) in Queensland (downloaded from the *Atlas of Living Australia*) demonstrating the gap in records between sympatric and allopatric populations of both species. Inset map shows area of sampling with respect to Australia.

distinguished by cranial characters, karyotypes, and genetic data (Watts & Aslin, 1981; Baverstock *et al*., 1986; Vasquez-Dominguez *et al*., 2001; Robins *et al*., 2007) indicating that they are genetically isolated biological species. Given that the species are genetically isolated but externally similar in phenotype, we anticipate that they are likely to use non-visual signals such as odorants in premating isolation (Panhuis *et al*., 2001; Zozaya *et al*., 2019).

In this study, we first used allozyme data to confirm the absence of gene flow between sympatric subspecies of *Rattus fuscipes* and *Rattus leucopus.* We then tested if the two species can be distinguished by the chemical composition of their preputial glands using extracts from wild-caught individuals. We compared compositional differences between species, between sympatric subspecies, and between allopatric conspecific subspecies to identify

putative compounds associated with species' boundaries. We also tested if sympatric subspecies (*Rfc* and *Rlc*) differ in the complexity of the chemical composition of their preputial glands compared to allopatric subspecies (*Rfa* and *Rll*). We predicted that if preputial gland odorants help the species to differentiate each other, then we would observe more complex chemical signatures in sympatric species versus allopatric subspecies and populations.

Methods

Specimens and tissue samples

Specimens were collected from four subspecies representing sympatric and allopatric populations of *R. fuscipes* and *R. leucopus*. *R. fuscipes coracius* (*Rfc*) and *R. leucopus cooktownensis* (*Rlc*) have sympatric distributions in the Wet Tropics region between Cooktown and Townsville, Queensland, Australia whereas *R. fuscipes assimilis* (*Rfa*) and *R. leucopus leucopus* (*Rll*) are from allopatric populations to the south and north respectively (Fig. 1). All populations sampled were from wet sclerophyll rainforest. Samples of *Rfc, Rfa,* and *Rlc* were collected over two seasons, February–March and July–August 2007, whereas all *Rll* were collected in August 2007. Heart, kidney, muscle, spleen, and preputial glands were collected in the field and stored in liquid nitrogen immediately after euthanizing vouchered specimens. All tissues were stored at −70° C until being shipped on dry ice to relevant labs for analyses. All samples were collected under permits from the Queensland Environmental Protection Agency (WITK04115806) following procedures authorized by the Southern Cross University Animal Care and Ethics Committee (permit 06/21). All tissues and voucher specimens were deposited in the Queensland Museum (Table S1, Rowe *et al.*, 2020).

Allozyme analyses

Frozen subsamples of heart, kidney, muscle, and spleen from 166 specimens (13 *Rfa*, 68 *Rfc*, 64 *Rlc*, and 21 *Rll*) were sent to the South Australian Museum for allozyme analysis, completed in 2007 (Table S1, Rowe *et al.*, 2020). Homogenates of each tissue were subjected to allozyme electrophoresis on cellulose acetate gels following procedures described previously (Richardson *et al*., 1986). We scored alleles for the following eighteen allozyme loci shown by Baverstock *et al*. (1986) to be informative in diagnosing the relevant species and subspecies: *Alb*, *Dia*, *Got-1*, *Got-2*, *Gpi*, *Gus*, *Idh-2*, *Ldh-1*, *Me*, *Mpi*, *Np*, *Pep-C2*, *6Pgd*, *Pgm-1*, *Pgm-2*, *Pk-3*, *Sod-1*, and *Sordh*. Details of enzyme and locus abbreviations, enzyme commission numbers, electrophoretic conditions, and stain recipes are provided in Richardson *et al*. (1986). We used the allozyme results to test for gene flow among sympatric taxa using the program Structure v2.3.3. We set the number of populations to 4 to reflect the four subspecies in our study but did not assign individuals to subspecies. We included admixture in model runs and ran 1,000,000 MCMC cycles, with the first 10,000 cycles discarded as burn-in.

Gas chromatography-mass spectrometry (GC-MS) Analysis of Preputial Glands

We used 32 preputial glands for chemical analysis. Glands were collected from eight males from each of the subspecies *Rfa, Rfc, Rlc,* and *Rll*. All but two samples (1 *Rll* and 1 *Rfc*) were collected from scrotal (i.e. reproductive) males. Whole, frozen preputial glands were shipped to the Institute for Pheromone Research at Indiana University (Bloomington, Indiana, USA) in 2009 where they were processed immediately. Frozen preputial glands were weighed and homogenized with a mortar and pestle on liquid nitrogen. Volatile organic compounds (VOCs) were extracted from each of the resulting homogenates using the sorptive extraction method with a polydimethylsiloxane (PDMS) polymer coated magnetic stir bar as described previously (Pohorecky *et al*., 2008; Baltussen *et al*., 1999; Soini *et al*., 2005). Briefly, the homogenized tissue (about 100 mg) was rinsed into the 20 mL glass scintillation vial (a tin foil lined cap) with 2.0 mL OmniSolv™ water (EMD Chemicals Inc., Billerica, Massachusetts, USA) and 500 µL of ethanol was added (100%, Pharmco-Aaper, Brookfield, Connecticut, USA). As an internal standard, 80 ng of 7-tridecanone (Sigma-Aldrich) in 10 µL of methanol (Baker Analyzed, Mallinckrodt Baker Inc., Phillipsburg, New Jersey, USA) was added to the vial. VOCs were extracted with a Twister™ stir bar (Gerstel GmbH, Mülheim an der Ruhr, Germany, 10 × 0.5 mm polydimethylsiloxane) by stirring at 800+ rpm for 60 min (15-place stir plate Variomag Multipoint HP15, H+P Labortechnic, Oberschleissheim, Germany). The stir bar was then rinsed with OmniSolv™ water, dried gently with a lint-free paper tissue (Kimwipes, Kimberly-Clark, Roswell, Georgia, USA) and placed in a Thermal Desorption Autosampler and Cooled Injection System (TDSA-CIS 4 from Gerstel GmbH) connected to an Agilent 6890N gas chromatograph—5973iMSD mass spectrometer (Agilent Technologies, Inc., Wilmington, Delaware, USA).

Splitless mode was used for thermal desorption with a temperature program of 20°C for 0.5 min, then a 60°C/ min increase up to 270°C for 8 min. The transfer line temperature was set at 280°C and the CIS was cooled using liquid nitrogen to −80°C. For the sample introduction into the GC-MS, the CIS was heated at 12°C/s to 280°C and held for 8 min. Solvent vent mode was used for the CIS inlet with a vent pressure of 8.0 psi, a vent flow of 50 mL/min, and a purge flow of 50 mL/min. The gas chromatograph (GC) separation capillary was a DB-5MS (30 m × 0.25 mm, i.d., 0.25 µm film thickness) from Agilent, and the GC helium carrier gas head pressure was 8.0 psi at a constant 1.1 mL/ min flow. The GC oven temperature program started at 40°C for 5 min, then increased at 2°C/ min to 200°C and was held for 15 min. For the mass spectrometer (MS), positive electron ionization (EI) mode at 70eV was used with a scanning rate of 2.47 scans/s and mass range of 41–350 amu. The mass spectrometric detector (MSD) transfer line temperature was 280°C, the ion source was 230°C, and quadrupole temperature was set at 150°C.

Compounds were positively or tentatively identified by matching retention times and mass spectra with standard

compounds when available from Sigma-Aldrich Chemical Co. and with spectra through NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.0a, 2002). Presence of sulphur and nitrogen in the identified compounds was verified by using the element-specific Agilent 6890 gas-chromatography—G2350A atomic emission detector (GC-AED) system (Agilent Technologies, Inc., Wilmington, Delaware, USA) under the conditions described previously (Novotny *et al*., 2007).

Statistical analyses of preputial gland chemical composition

We first used the chemical analysis to test if species and subspecies could be distinguished by the chemical composition of their preputial glands. For statistical analyses of preputial gland chemical composition, we included only identified and quantitated compounds. We conducted analyses on two sets of compounds. The first set included all identified compounds, hereafter referred to as "total". The second set, hereafter referred to as "subset", included compounds proposed to have roles in chemical communication based on studies of other mammalian species, i.e. thiazolines, carboxylic acids, and two geranyl-related compounds (Schwende *et al*., 1986; reviewed in Petrulis, 2013). To visualize the dissimilarity in preputial gland chemical composition among species, subspecies, and individuals we used a non-metric multidimensional scaling (MDS) approach described previously (Zimmerman *et al*., 2009; Weber *et al*., 2016). For each sample we calculated the relative abundance of each compound by dividing its absolute peak area by the sum of all peak areas for that sample. We transformed these percent values with a square-root. For the subset analysis, we retained percent values calculated from the total pool of compounds. From these transformed values, representing relative abundance of each chemical compound in each individual, we calculated a Bray-Curtis index of dissimilarity among pairs of individuals. The Bray-Curtis index considers only compounds shared between each pair of individuals. We used MDS in the R (v. 3.6.1, R Core Team 2019) package "vegan" (v. 2.5–6, Oksanen *et al*. 2019) to reduce the dimensionality of this matrix of Bray-Curtis distances. We used 1000 iterations with the "metaMDS" function to identify the scores with the minimum stress values. To visualize the dissimilarity among individuals we plotted the first two dimensions of the resulting MDS. We used an analysis of similarity (ANOSIM) using the "anosim" function in "vegan" to test if MDS-ordinated chemical composition is significantly different among species and subspecies relative to variation among individuals within groups. To determine which chemical compounds most-differentiate species and subspecies we performed a SIMPER (similarity percentage) analysis using the "simper" function in "vegan".

To examine if the complexity of preputial gland chemical composition varies among all subspecies and between sympatric and allopatric subspecies of *R. fuscipes* and *R. leucopus* we quantified two variables per individual, (1) the number of chemical compounds detected, and (2) the total abundance of chemical compounds. We defined the number of chemical compounds per individual as a count of detected compounds regardless of peak area. We defined the total abundance of chemical compounds per individual as the sum of the peak areas of all compounds. We tested for an overall significant difference among the four subspecies using a Kruskal-Wallis H test. We then tested for a significant increase in the count and abundance of chemical compounds in sympatric subspecies compared to allopatric subspecies with a one-way Mann-Whitney U test. Both tests were performed using the R Core "stats" package.

Results

Allozyme analyses

We recovered no shared polymorphism between *R. fuscipes* and *R. leucopus* for 10 out of the 18 allozyme loci screened (Table 1). Not surprisingly, Structure analyses found no evidence of gene flow among species in sympatry. With K = 4 populations, Structure clearly separated the two species and most individuals of the four subspecies (Fig. 2). All *Rfa* and *Rfc* individuals were correctly classified to their respective populations with high probability, whereas a few *Rlc* and one *Rll* individuals were close to equivocal with regard to population assignment suggesting the retention of shared polymorphism between these two allopatric subspecies.

Figure 2. Structure plot of allozyme variation among four subspecies of Australian *Rattus*. Each of the 166 samples analysed in this study (13 *Rfa*, 67 *Rfc*, 65 *Rlc*, 21 *Rll*) is represented by a vertical bar shaded based on the likelihood of assignment to one of four populations. Individual samples are not distinguishable where they share a high likelihood of assignment to the same population (e.g., all *Rfa* samples). Plot demonstrates lack of gene flow between species in sympatry (*Rfc* and *Rlc*) with no mixed likelihood between species. Limited gene flow (or shared polymorphism) among subspecies, *Rlc* and *Rll*, are evident in bars with mixed shading.

Table 1. Allozyme profiles at 18 loci for the four *Rattus* taxa surveyed. For polymorphic loci, allozyme frequencies for all but the rarest are expressed as percentages and shown as superscripts. Loci that display fixed differences between species, *R. leucopus* and *R. fuscipes* are highlighted with an asterisk. Sample sizes per taxon are shown in parentheses.

locus	*R. l. leucopus* (21)	*R. l. cooktownensis* (65)	*R. f. coracius* (67)	*R. f. assimilis* (13)
Alb	b^{52},c	b^{98},c	b^{99},a	b^{92},c
Dia	a^{64},b	a	a^{98},b	a
Got-1	c	c	c^{72},a^{27},b	c
*Got-2**	a	a	b	b
*Gpi**	a^{55},c	a	b	b
Gus	b	b^{99},c	c^{93},d^{6},b	c^{73},a
*Idh-2**	c	c	a^{60},b	a
*Ldh-1**	b	b	a	a
*Me**	b^{90},a	b^{98},c	d	d
*Mpi**	a	a	b	b
*Np**	c^{98},d	c^{98},d	b^{69},a	a
Pep-C2	a	b^{86},a	a	a
6Pgd	b^{93},d	b	b^{97},c	a
Pgm-1	c^{93},b	c^{99},d	b^{64},c^{20},a	c
Pgm-2	b^{90},a	b	b	b^{54},c
*Pk-3**	a	a^{98},c	b	b
*Sod-1**	b	b	a	a
*Sordh**	b	b^{99},c	a	a

Chemical composition of preputial glands

Preputial gland volatile compound profiles from male *R. fuscipes* and *R. leucopus* determined by GC-MS recovered 278 volatile organic compounds (VOCs; data not shown). Among these 278 compounds, we positively or tentatively identified 120 compounds (43%), including linear ketones, a series of ten methylketones from 2-heptanone to 2-heptadecanone, aldehydes, alcohols, aliphatic acids, esters, hydrocarbons, cholesterol, terpenes, terpene alcohols, seven thiazolines, and seven carboxylic acids. Out of these 120 compounds, we quantitated 80 consistently appearing compounds (29% of all compounds) by measuring their peak areas and normalizing them by the peak area of the internal standard (Table S2, Rowe *et al.*, 2020). We refer to these 80 quantitated compounds for subsequent analysis of differences between species and among subspecies. We identified the seven thiazoline compounds as 2-methylthiazoline, 2-ethylthiazoline, 2-isopropylthiazoline, 2-propylthiazoline, 2-sec-butylthiazoline, 2-isobutylthiazoline and 2-butylthiazoline (Table 2 and Table S2). The thiazoline chemical structures found in this study are presented in Fig. 3. We also identified the seven carboxylic acid compounds as dodecanoic acid, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, linoleic acid, oleic acid, octadecanoic acid. Finally, we partially identified, three geranyl-related sesquiterpenes at retention times of 40.83 min, 41.62 min and 42.27 min that are potentially important in chemical communication similar to farnesenes (Harvey *et al*., 1989; Novotny *et al*., 1980; Pohorecky *et al*., 2008).

Between species, we detected eight quantitated compounds exclusively in *R. leucopus* (Table 2; Table S2;

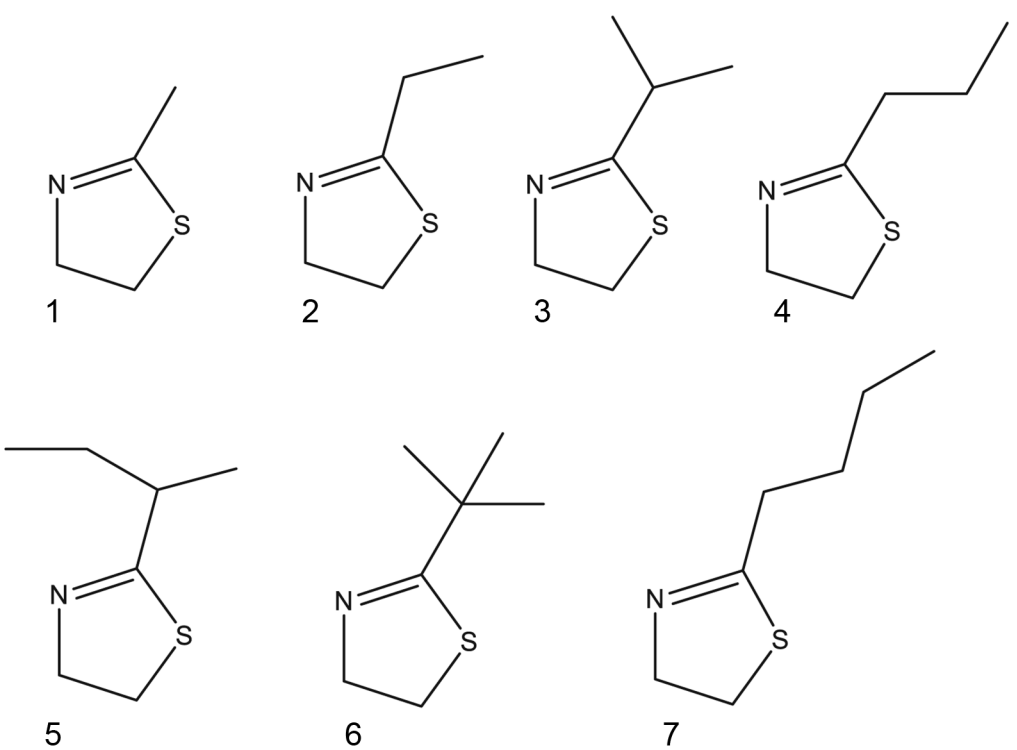

Figure 3. Chemical structures of seven thiazoline compounds identified from preputial glands of *Rattus fuscipes* and *R. leucopus* in this study. Numbers refer to (*1*) 2-methylthiazoline; (*2*) 2-ethylthiazoline; (*3*) 2-isopropylthiazoline; (*4*) 2-propylthiazoline; (*5*) 2-sec-butylthiazoline; (*6*) 2-isobutylthiazoline (detected only in *R. l. leucopus*); and (*7*) 2-butylthiazoline.

Table 2. Means ± standard deviations for relative proportion of each of the 80 quantitated chemical compounds from preputial gland extracts of *R. fuscipes, R. leucopus,* and subspecies. Molecular weight (*MW*), quantification single ion (*ion*), and retention time (*min*) presented for each compound. For ion quantification, *TIC* indicates that peak areas were calculated from the total ion chromatogram, whereas numerical ion values are indicative of peak areas defined by post-run extracted single ion chromatograms (SIC). Compounds included in our subset analyses our indicated with an asterisk.

		chemical compound		species		subspecies			
MW	ion	name	min	*R. fuscipes*	*R. leucopus*	*R.f.assimilis*	*R.f.coracius*	*R.l.cooktownensis*	*R.l.leucopus*
98	TIC	Furfuryl alcohol	9.48	0.183 (±0.10)	0.066 (±0.03)	0.220 (±0.13)	0.146 (±0.04)	0.053 (±0.01)	0.079 (±0.04)
101	60	2-Methylthiazoline*	10.56	0.049 (±0.02)	0.024 (±0.01)	0.063 (±0.01)	0.035 (±0.01)	0.021 (±0.01)	0.027 (±0.01)
114	58	2-Heptanone	11.31	0.014 (±0.01)	0.026 (±0.01)	0.012 (±0.01)	0.015 (±0.01)	0.020 (±0.00)	0.033 (±0.02)
84	TIC	Crotonolactone	12.5	0.079 (±0.04)	0.023 (±0.02)	0.095 (±0.05)	0.063 (±0.02)	0.017 (±0.01)	0.028 (±0.02)
115	60	2-Ethylthiazoline*	16.2	0.044 (±0.01)	0.031 (±0.01)	0.048 (±0.01)	0.041 (±0.01)	0.024 (±0.00)	0.039 (±0.01)
140	55	1-Decene	18.03	0.052 (±0.02)	0.024 (±0.01)	0.069 (±0.01)	0.036 (±0.01)	0.015 (±0.00)	0.034 (±0.01)
129	60	2-Isopropylthiazoline*	19.59	0.069 (±0.04)	0.101 (±0.05)	0.039 (±0.01)	0.099 (±0.04)	0.052 (±0.02)	0.151 (±0.02)
138	TIC	1-para-Menthene	19.99	0.057 (±0.03)	0.037 (±0.02)	0.073 (±0.02)	0.041 (±0.02)	0.036 (±0.01)	0.037 (±0.02)
136	TIC	Limonene	20.41	0.075 (±0.03)	0.037 (±0.01)	0.098 (±0.01)	0.052 (±0.01)	0.03 (±0.01)	0.044 (±0.01)
129	60	2-Propylthiazoline*	22.49	0.015 (±0.01)	0.014 (±0.01)	0.005 (±0.01)	0.026 (±0.01)	0.009 (±0.00)	0.02 (±0.01)
142	58	4-Methyl-2,6-heptanedione?	22.46	0.012 (±0.01)	0.042 (±0.02)	0	0.025 (±0.01)	0.027 (±0.00)	0.056 (±0.01)
126	TIC	2-Octenal	22.7	0.123 (±0.07)	0.041 (±0.02)	0.164 (±0.08)	0.083 (±0.04)	0.025 (±0.01)	0.057 (±0.02)
120	TIC	Acetophenone	22.93	0.104 (±0.05)	0.047 (±0.02)	0.144 (±0.05)	0.064 (±0.02)	0.032 (±0.01)	0.062 (±0.01)
142	TIC	7-Methyl-2-octanone	23.57	0	0.003 (±0.01)	0	0	0.006 (±0.02)	0
142	58	2-Nonanone	25.19	0.112 (±0.06)	0.311 (±0.06)	0.070 (±0.02)	0.154 (±0.06)	0.284 (±0.05)	0.338 (±0.05)
143	60	2-sec-Butylthiazoline*	25.89	0.180 (±0.06)	0.078 (±0.03)	0.173 (±0.05)	0.187 (±0.07)	0.056 (±0.01)	0.100 (±0.02)
144	TIC	2-Nonanol	26.01	0.050 (±0.06)	0.021 (±0.02)	0	0.099 (±0.05)	0.030 (±0.02)	0.013 (±0.03)
143	60	2-Isobutylthiazoline*	26.1	0	0.024 (±0.03)	0	0	0	0.047 (±0.01)
142	TIC	Nonanal	26.14	0.176 (±0.08)	0.048 (±0.03)	0.226 (±0.06)	0.126 (±0.06)	0.040 (±0.02)	0.056 (±0.03)
156	TIC	C9 2,x-Diketone A branched	29.25	0.024 (±0.03)	0.034 (±0.03)	0	0.047 (±0.02)	0.050 (±0.02)	0.019 (±0.02)
156	58	C9 2,x-Diketone B	29.76	0.005 (±0.01)	0.032 (±0.02)	0	0.010 (±0.01)	0.013 (±0.00)	0.050 (±0.01)
	TIC	C9 2,x-Diketone B	29.76	0.015 (±0.03)	0.066 (±0.04)	0	0.030 (±0.03)	0.030 (±0.00)	0.102 (±0.03)
143	60	2-Butylthiazoline*	29.85	0.011 (±0.01)	0.007 (±0.00)	0.005 (±0.01)	0.016 (±0.00)	0.005 (±0.00)	0.009 (±0.00)
140	TIC	2-Nonenal	30.01	0.077 (±0.05)	0.040 (±0.03)	0.087 (±0.07)	0.068 (±0.03)	0.023 (±0.01)	0.057 (±0.03)
156	58	C9 2,x-Diketone C	30.13	0.025 (±0.02)	0.033 (±0.01)	0.011 (±0.00)	0.038 (±0.01)	0.032 (±0.01)	0.034 (±0.01)
150	TIC	Ethyl benzoate	30.55	0.105 (±0.04)	0.033 (±0.01)	0.135 (±0.04)	0.076 (±0.02)	0.027 (±0.01)	0.039 (±0.01)
156	58	2-Decanone	32.37	0.062 (±0.06)	0.145 (±0.03)	0.020 (±0.03)	0.104 (±0.04)	0.140 (±0.02)	0.151 (±0.03)
158	TIC	2-Decanol	33.14	0.040 (±0.05)	0.022 (±0.01)	0	0.08 (±0.04)	0.021 (±0.01)	0.022 (±0.02)
156	TIC	Decanal	33.34	0.148 (±0.08)	0.061 (±0.03)	0.191 (±0.06)	0.105 (±0.06)	0.041 (±0.01)	0.080 (±0.02)
152	TIC	Neral	35.38	0.075 (±0.04)	0.033 (±0.02)	0.110 (±0.01)	0.041 (±0.01)	0.022 (±0.01)	0.043 (±0.03)
154	55	3-Decen-2-one	35.56	0.005 (±0.01)	0.022 (±0.01)	0.004 (±0.01)	0.006 (±0.00)	0.003 (±0.01)	0.013 (±0.01)
170	58	C10 2,x-Diketone A	36.83	0.033 (±0.04)	0.080 (±0.03)	0	0.065 (±0.02)	0.060 (±0.02)	0.099 (±0.03)
152	TIC	Geranial	37.49	0.149 (±0.10)	0.098 (±0.06)	0.195 (±0.12)	0.102 (±0.03)	0.080 (±0.04)	0.116 (±0.07)
168	TIC	Undecen-2-one A	37.83	0.217 (±0.10)	0.123 (±0.05)	0.144 (±0.03)	0.290 (±0.10)	0.167 (±0.04)	0.079 (±0.03)
168	TIC	Undecen-2-one B	38.06	0.029 (±0.04)	0.064 (±0.02)	0	0.059 (±0.03)	0.066 (±0.01)	0.062 (±0.03)
168	TIC	Undecen-2-one C	38.62	0.263 (±0.17)	0.156 (±0.07)	0.117 (±0.06)	0.408 (±0.11)	0.170 (±0.04)	0.141 (±0.08)
168	TIC	C10 2,x-Diketone B	39.03	0.195 (±0.23)	0.636 (±0.18)	0	0.390 (±0.17)	0.656 (±0.18)	0.617 (±0.18)
170	58	2-Undecanone	39.4	0.110 (±0.10)	0.371 (±0.07)	0.041 (±0.06)	0.179 (±0.09)	0.378 (±0.06)	0.364 (±0.08)
172	TIC	2-Undecanol	40.04	0.080 (±0.09)	0.083 (±0.04)	0	0.160 (±0.06)	0.077 (±0.04)	0.089 (±0.03)
166	TIC	69-98-41-81: A geranyl-related ketone	40.83	0.300 (±0.13)	0.058 (±0.04)	0.419 (±0.03)	0.181 (±0.05)	0.061 (±0.02)	0.055 (±0.06)
166	TIC	69-41-137-95-108--geranyl-related*	41.62	0.087 (±0.05)	0.002 (±0.01)	0.133 (±0.02)	0.040 (±0.01)	0	0.004 (±0.01)
166	TIC	69-41-137-95-108--geranyl-related*	42.27	0.060 (±0.04)	0	0.097 (±0.01)	0.023 (±0.02)	0	0
168	55	3-Undecen-2-one	42.4	0.022 (±0.01)	0.078 (±0.04)	0.019 (±0.01)	0.024 (±0.01)	0.116 (±0.02)	0.040 (±0.02)
184	TIC	C11 2,x-Diketone A branched	42.75	0.004 (±0.01)	0.025 (±0.02)	0	0.009 (±0.01)	0.038 (±0.02)	0.011 (±0.01)
184	58	C11 2,x-Diketone B	43.51	0	0.017 (±0.01)	0	0	0.009 (±0.00)	0.024 (±0.00)
184	58	C11 2,x-Diketone C	43.91	0.014 (±0.02)	0.030 (±0.01)	0	0.027 (±0.02)	0.030 (±0.01)	0.029 (±0.01)
182	43	x-Dodecen-2-one	44.3	0.036 (±0.03)	0.028 (±0.01)	0.022 (±0.01)	0.050 (±0.03)	0.038 (±0.01)	0.018 (±0.01)
	TIC	x-Dodecen-2-one	44.3	0.113 (±0.10)	0.101 (±0.04)	0.048 (±0.07)	0.178 (±0.09)	0.132 (±0.03)	0.069 (±0.02)
172	55	1-Undecanol	44.64	0.021 (±0.01)	0.013 (±0.01)	0.026 (±0.02)	0.017 (±0.01)	0.009 (±0.00)	0.017 (±0.02)
184	58	2-Dodecanone	45.84	0.014 (±0.02)	0.062 (±0.01)	0	0.027 (±0.02)	0.061 (±0.01)	0.062 (±0.01)
194	TIC	Geranylacetone	49.09	0.156 (±0.08)	0.064 (±0.02)	0.227 (±0.03)	0.085 (±0.05)	0.049 (±0.01)	0.079 (±0.02)
198	58	C12 2,x-Diketone A	49.87	0.005 (±0.01)	0.022 (±0.01)	0	0.009 (±0.01)	0.020 (±0.00)	0.024 (±0.01)
196	58	Tridecen-2-one A	50.46	0.013 (±0.01)	0.028 (±0.01)	0.002 (±0.00)	0.024 (±0.01)	0.038 (±0.01)	0.018 (±0.01)
186	55	1-Dodecanol	50.97	0.042 (±0.02)	0.028 (±0.01)	0.044 (±0.03)	0.040 (±0.01)	0.026 (±0.01)	0.030 (±0.01)
196	TIC	Tridecen-2-one B	51.19	0.027 (±0.04)	0.088 (±0.04)	0	0.053 (±0.03)	0.116 (±0.03)	0.059 (±0.04)
198	58	2-Tridecanone	52.15	0.033 (±0.01)	0.152 (±0.03)	0.028 (±0.01)	0.038 (±0.02)	0.163 (±0.04)	0.141 (±0.02)
200	TIC	2-Tridecanol	52.72	0.012 (±0.02)	0.054 (±0.02)	0	0.025 (±0.03)	0.056 (±0.02)	0.052 (±0.03)
194	TIC	6,10-Dimethyl-3-undecen-2-one?	53.57	0.005 (±0.01)	0.090 (±0.04)	0	0.010 (±0.01)	0.116 (±0.02)	0.063 (±0.04)
222	TIC	Z-Nerolidol	54.28	0.050 (±0.03)	0.010 (±0.01)	0.035 (±0.02)	0.066 (±0.02)	0.009 (±0.01)	0.012 (±0.01)
196	55	3-Tridecen-2-one	54.95	0.015 (±0.01)	0.014 (±0.01)	0.019 (±0.01)	0.011 (±0.01)	0.016 (±0.00)	0.012 (±0.01)
222	TIC	E-Nerolidol	55.9	0.073 (±0.02)	0.040 (±0.01)	0.087 (±0.02)	0.058 (±0.01)	0.032 (±0.01)	0.049 (±0.01)
200	TIC	Dodecanoic acid*	56.27	0.245 (±0.14)	0.062 (±0.03)	0.319 (±0.15)	0.171 (±0.09)	0.057 (±0.02)	0.066 (±0.04)
210	55	x-Tetradecen-2-one	56.91	0	0.009 (±0.01)	0	0	0.014 (±0.00)	0.004 (±0.01)
212	58	2-Tetradecanone	58.05	0	0.025 (±0.01)	0	0	0.031 (±0.01)	0.019 (±0.01)
182	TIC	Benzophenone	59.21	0.017 (±0.02)	0.022 (±0.02)	0.031 (±0.03)	0.003 (±0.01)	0.011 (±0.00)	0.033 (±0.02)
222	TIC	x,y-Pentadecadien-2-one	61.41	0.005 (±0.02)	0.100 (±0.03)	0	0.010 (±0.03)	0.119 (±0.02)	0.081 (±0.02)
226	TIC	2-Pentadecanone (branched)	61.66	0	0.031 (±0.01)	0	0	0.032 (±0.01)	0.030 (±0.02)
224	TIC	Pentadecen-2-one A	62.07	0.003 (±0.01)	0.046 (±0.02)	0	0.007 (±0.02)	0.054 (±0.02)	0.037 (±0.02)
224	TIC	Pentadecen-2-one B	62.39	0.034 (±0.05)	0.104 (±0.06)	0	0.069 (±0.04)	0.145 (±0.05)	0.062 (±0.04)
226	58	2-Pentadecanone	63.7	0.020 (±0.01)	0.071 (±0.03)	0.018 (±0.01)	0.022 (±0.00)	0.088 (±0.04)	0.054 (±0.01)
228	TIC	2-Pentadecanol	64.23	0	0.021 (±0.02)	0	0	0.031 (±0.02)	0.012 (±0.02)
228	60	Tetradecanoic acid*	67.25	0.078 (±0.03)	0.032 (±0.02)	0.090 (±0.02)	0.065 (±0.03)	0.021 (±0.01)	0.044 (±0.02)
240	TIC	2-Hexadecanone	69.05	0.002 (±0.01)	0.019 (±0.02)	0	0.004 (±0.01)	0.030 (±0.01)	0.008 (±0.02)
242	60	Pentadecanoic acid*	72.34	0.098 (±0.03)	0.025 (±0.02)	0.119 (±0.03)	0.077 (±0.02)	0.014 (±0.00)	0.037 (±0.01)
252	TIC	A heptadecen-2-one	72.77	0	0.010 (±0.02)	0	0	0.019 (±0.02)	0
254	TIC	2-Heptadecanone	74.19	0.014 (±0.03)	0.035 (±0.02)	0.009 (±0.03)	0.019 (±0.04)	0.042 (±0.02)	0.029 (±0.03)
256	60	Hexadecanoic acid*	77.5	0.230 (±0.08)	0.092 (±0.05)	0.276 (±0.04)	0.183 (±0.08)	0.056 (±0.01)	0.128 (±0.04)
280	55	Linoleic acid*	85	0.131 (±0.06)	0.068 (±0.04)	0.134 (±0.06)	0.128 (±0.07)	0.042 (±0.01)	0.093 (±0.05)
282	55	Oleic acid*	85.31	0.158 (±0.08)	0.063 (±0.04)	0.188 (±0.09)	0.128 (±0.07)	0.040 (±0.01)	0.087 (±0.05)
284	55	Octodecanoic acid*	86.58	0.069 (±0.03)	0.028 (±0.01)	0.082 (±0.02)	0.056 (±0.03)	0.019 (±0.00)	0.037 (±0.01)

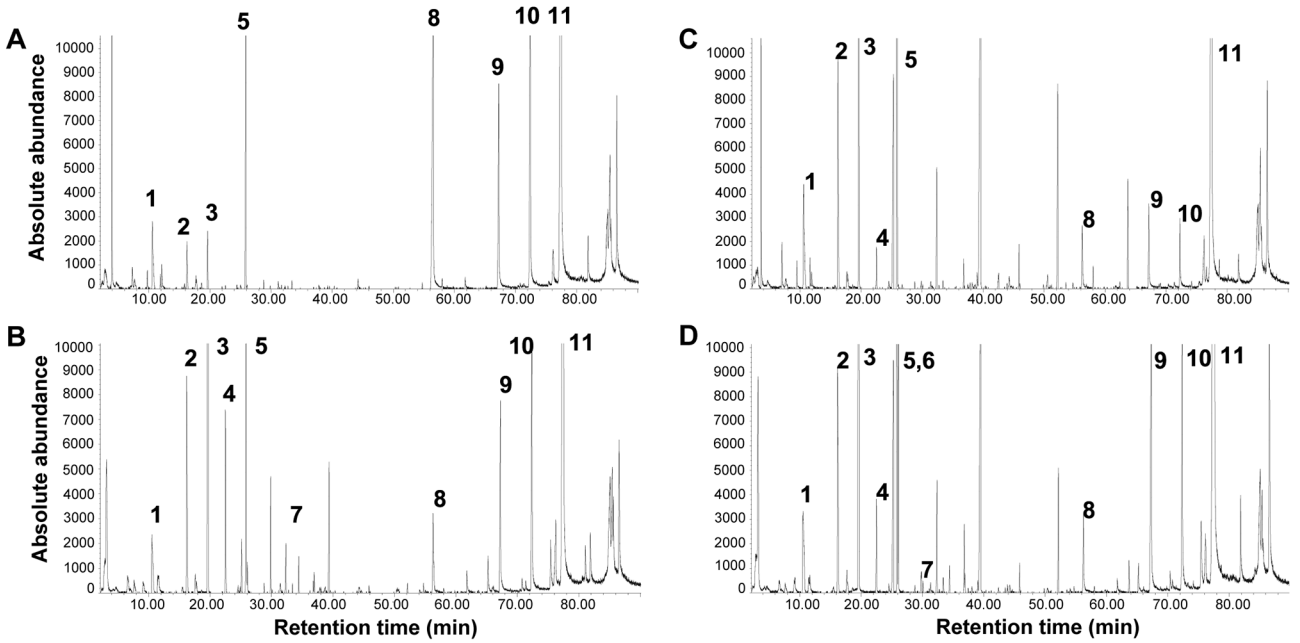

Figure 4. Representative ion m/z 60 trace with post-run selected ion chromatograms for thiazolines and carboxylic acids from preputial gland extracts of (*A*) *Rattus fuscipes assimilis* (QMJM 19152); (*B*) *R. fuscipes coracius* QMJM 19100; (*C*) *R. leucopus cooktownensis* QMJM 19131; and (*D*) *R. leucopus leucopus* QMJM 19060. Numbers above peaks identify specific compounds: (*1*) 2-methylthiazoline, 10.56 min; (*2*) 2-ethylthiazoline, 16.04 min; (*3*) 2-isopropylthiazoline, 19.59 min; (*4*) 2-propylthiazoline, 22.49 min; (*5*) 2-sec-butylthiazoline (SBT), 25.89 min; (*6*) 2-isobutylthiazoline, 26.10 min; (*7*) 2-butylthiazoline, 29.85 min; (*8*) dodecanoic acid, 56.28 min; (*9*) tetradecanoic acid, 67.25 min; (*10*) pentadecanoic acid, 72.34 min; and (*11*) hexadecanoic acid, 77.50 min.

C11 2,x-diketone B, x-tetradecen-2-one, 2-tetradecanone, 2-pentadecanone (branched), 2-pentadecanol, 7-methyl-2-octanone, a heptadecen-2-one, and 2-isobutylthiazoline). One of these, 7-methyl-2-octanone, was only detected in a single sample. We also detected three compounds that were common across both *R. leucopus* subspecies but each was detected in only a single *R. fuscipes* sample and all in *Rfc* (x,y-pentadecadien-2-one, 2-hexadecanone, and pentadecen-2-one A). Of the eight compounds exclusive to *R. leucopus*, two were detected only in *Rlc* (7-methyl-2-octanone, a heptadecen-2-one) and one only in *Rll* (2-isobutylthiazoline). In contrast, we detected two geranyl-related sesquiterpene compounds at retention times 41.62 min and 42.27 min that, with the exception of a low abundance detection in a single *Rll* sample, were exclusive to *R. fuscipes*. Sixteen compounds were detected in all subspecies except *Rfa* (4-methyl-2-6-heptanedione, C9-2-x-diketone-A (branched), C9-2-x-diketone-B, C9-2-x-diketone-B, 2-decanol, C10-2-x-diketone-A, undecen-2-one-B, C10-2-x-diketone-B, 2-undecanol, C11-2-x-diketone-C, 2-dodecanone, C12-2-x-diketone-A, tridecen-2-one-B, 2-tridecanol, 6-10-dimethyl-3-undecen-2-one, pentadecen-2-one-B). Among thiazoline compounds, 2-methylthiazoline, 2-ethylthiazoline, 2-isopropylthiazoline, 2-propylthiazoline, 2-butylthiazoline, and 2-sec-butylthiazoline were found in all subspecies of *R. leucopus* and *R. fuscipes*. We detected 2-isobutylthiazoline in every sample of *Rll* but did not detect it in any other subspecies. Comparative ion m/z 60 chromatograms for seven thiazolines and four carboxylic acids in representative samples of each subspecies with similar preputial gland masses (each about 100 mg) show substantial qualitative differences between species (Fig. 4).

Extensive quantitative differences in the composition of VOCs from preputial glands separated both species and subspecies of *R. fuscipes* and *R. leucopus* (Fig. 5). The two-dimensional multidimensional scaling (MDS) plot of the proportional abundance of total VOCs clearly separated species and subspecies with no overlap (Fig. 5A). The ANOSIM permutation analyses confirmed a significant effect of both species ($R = 0.75$, $p < 0.001$) and subspecies ($R = 0.88$, $p < 0.001$) on composition similarity compared to variation among individuals within species or subspecies (Fig. 5B). Our analysis of the subset of VOCs (thiazolines, carboxylic acids, and geranyl-related) produced similar patterns. Both species and subspecies are clearly separated in two-dimensional MDS space and outliers are less evident (Fig. 5C). However, sample points are more continuously distributed with less disjunct gaps among species and subspecies. This is reflected in the ANOSIM analyses of the subset data, which found a significant effect of both species ($R = 0.69$, $p < 0.001$) and subspecies ($R = 0.86$, $p < 0.001$) but with somewhat lower R values than with the total compound data (Fig. 5D). The SIMPER analyses identified 30 compounds that cumulatively explain 70% of the variation between species (Table 3). Among subspecies ten of these compounds explain a greater proportion of difference between sympatric taxa (*Rfc*/*Rlc*) than between allopatric, conspecific subspecies (*Rfc/Rfa* or *Rlc/Rll*). The SIMPER analyses found that four of the 17 compounds in our subset data contributed to differences among species or subspecies, whereas the remaining seven did not.

We also recovered a consistent pattern of increased chemical complexity in both species in sympatry. The

Table 3. SIMPER analysis of relative contributions of chemical compounds to MDS differences between species and subspecies of *Rattus fuscipes* and *R. leucopus.* Compounds shown are those that contribute cumulatively 70% of the differences between species and are sorted by relative contribution to differences between species. Compounds with a greater relative contribution to differences between sympatric subspecies compared to allopatric, conspecific subspecies are highlighted in grey. Compounds included in our subset analyses are indicated with an asterisk. Superscript numbers indicate that a compound is depicted in Figs 3 or 4, and which figure.

		relative contribution to differences between		
compound	species *R. leucopus* and *R. fuscipes*	sympatric subspecies *Rlc* and *Rfc*	conspecifics *Rfc* and *Rfa*	conspecifics *Rlc* and *Rll*
C10 2,x-Diketone B	8.76%	7.22%	1.68%	5.19%
2-Undecanone	4.80%	4.47%	3.06%	2.10%
69-98-41-81: A geranyl-related ketone	4.52%	2.73%	4.88%	1.79%
2-Nonanone	3.65%	2.91%	1.82%	2.74%
Dodecanoic acid*,4	3.42%	2.59%	3.61%	1.30%
Undecen-2-one C	2.97%	5.33%	5.95%	2.93%
Hexadecanoic acid*,4	2.66%	2.90%	2.34%	2.68%
Nonanal	2.41%	1.97%	2.35%	1.15%
Octadecanoic acid*	2.33%	2.10%	2.54%	1.54%
Furfuryl alcohol	2.33%	2.10%	2.54%	1.54%
2-Tridecanone	2.18%	2.79%	0.37%	1.25%
Undecen-2-one A	2.06%	3.11%	3.17%	3.31%
Oleic acid	2.03%	2.01%	2.26%	2.14%
Geranylacetone	1.92%	1.09%	2.92%	1.09%
2-sec-Butylthiazoline (SBT)*,3,4	1.91%	2.97%	1.36%	1.67%
Geranial	1.80%	0.91%	2.96%	2.21%
x,y-Pentadecadien-2-one	1.75%	2.43%	0.21%	1.43%
Decanal	1.74%	1.47%	2.17%	1.49%
x-Dodecen-2-one	1.71%	1.95%	2.96%	2.39%
2-Decanone	1.66%	1.11%	1.75%	1.14%
2-Octenal	1.63%	1.44%	1.78%	1.23%
2-Undecanol	1.59%	2.18%	3.27%	1.44%
69-41-137-95-108: geranyl-related (41.62 m)*	1.57%	2.73%	1.92%	2.26%
6,10-Dimethyl-3-undecen-2-one	1.55%	2.40%	0.21%	2.26%
Pentadecen-2-one B	1.53%	1.81%	1.40%	3.29%
Linoleic acid*	1.47%	1.96%	1.44%	2.30%
Pentadecanoic acid*,4	1.35%	1.43%	0.97%	0.89%
Ethyl benzoate	1.34%	1.08%	1.31%	0.58%
Tridecen-2-one B	1.27%	1.42%	1.08%	2.28%
Acetophenone	1.15%	0.75%	1.68%	1.15%
total	71.04%	71.37%	65.96%	58.73%

average number of chemical compounds ($\chi^2 = 27.10$, $p < 0.001$) and the total abundance ($\chi^2 = 25.64$, $p < 0.001$) of chemical compounds were both significantly different among subspecies. On average, *Rlc* males had more chemical compounds and higher total compound abundance than all other subspecies (Fig. 6A,B). *Rfa* males in contrast had fewer numbers of compounds and lower total compound abundance than all other subspecies. The two sympatric taxa, *Rfc* and *Rlc*, both had significantly more compounds and significantly higher total compound abundance than their respective allopatric conspecific subspecies, *Rfa* (count, $Z = 63.5$, $p < 0.001$; abundance, $Z = 62$, $p < 0.001$) and *Rll* (count, $Z = 59.5$, $p = 0.002$; abundance, $Z = 64$, $p < 0.001$).

Discussion

We identified and quantitated 78 and 69 volatile organic compounds (VOC) from preputial glands of *Rattus leucopus* and *Rattus fuscipes,* respectively. Most of these VOCs have been frequently reported in different mammalian gland secretions in different combinations and at different concentration levels. Here, we identified seven thiazoline compounds that are rarely detected in mammals but have been previously linked to sexual and social interactions in rodents or other mammals. Two of these compounds, 2-isopropylthiazoline and 2-sec-butylthiazoline (SBT), are under endocrine control and have been found in high concentrations in the urine of male house mice, *Mus musculus* (Schwende *et al*., 1986; Novotny *et al*., 2007), where they are involved in intermale aggression and are attractive to females (Jemiolo *et al*., 1985; Novotny *et al*., 1985; Schwende *et al*., 1986; reviewed in Petrulis, 2013). The chirality of 2-sec-butylthiazoline (Novotny *et al*., 1995;

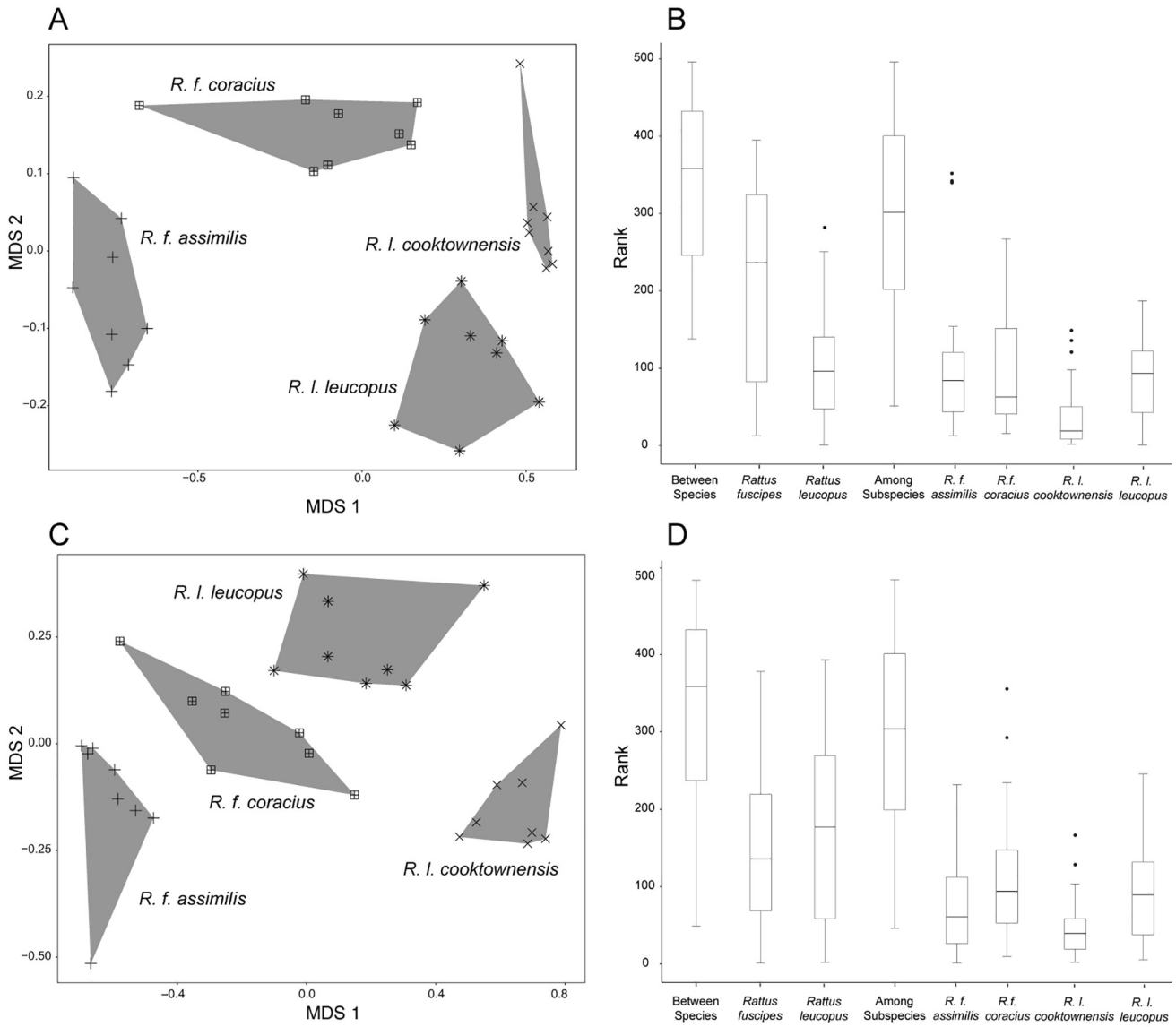

Figure 5. Variation in chemical composition of preputial glands among four subspecies of *Rattus fuscipes* and *R. leucopus*. (*A*) Two-dimensional representation of chemical composition among individuals based on non-metric multidimensional scaling of all 80 quantitated compounds showing separation of species and subspecies (grey polygons represent grouping of samples using convex hulls). (*B*) Anosim plot of total compounds showing greater variation between than within species and among than within subspecies. (*C*) Two-dimensional representation of chemical composition among individuals based on non-metric multidimensional scaling using subset of thiazoline, carboxylic acid, and sesquiterpene compounds showing separation of species and subspecies (grey polygons represent grouping of samples using convex hulls). (*D*) Anosim plot of subset of compounds showing greater variation between than within species and among than within subspecies.

Cavaggioni *et al*., 2003) may be important for efficient and specific binding to receptors. In *M. musculus*, racemic SBT activates vomeronasal neurons with high specificity, suggesting a primary role as a pheromone (Leinders-Zufall *et al*., 2000). Recently, SBT activity also has been linked to adult brain neurogenesis (Koyama *et al*., 2013; 2014) and induced cross-generational effects in *M. musculus* (Koyama *et al*., 2015) suggesting that this thiazoline is important in learning and the inheritance of learned behaviours, like mate choice. In *M. musculus*, SBT has been detected in preputial glands, urine, blood (Novotny *et al*., 2007) and saliva (Novotny & Soini, 2008) suggesting that it is a systemic metabolite. The biosynthetic pathway of SBT includes the amino acids isoleucine and cysteine as precursors (Novotny *et al*., 1995). SBT is also known to be produced by *M. musculus* under alarm conditions and to activate neurons of the Grueneberg ganglion involved in alarm response (Brechbühl *et al*., 2013). SBT is structurally similar to another thiazoline, 2,3,5-trimethyl-3-thiazoline (TMT) that is found in predator feces, induces an alarm response, and also activates neurons of the Grueneberg ganglions in rodents (Vernet-Maury, 1980; review by Fendt & Endres, 2008; Brechbühl *et al*., 2013). We also identified in *R. fuscipes* and *R. leucopus* two other thiazolines (2-isobutyl-1,3-thiazole and its 4,5-dihydro derivative) which are important in territorial marking in African antelopes (*Sylvicapra grimmia* and *Cephalophus natalensis*; Burger *et al*., 1988), and have not been reported previously in preputial gland secretions of rodents. The series of methylketones (2-ketones) identified in this study, including 2-heptanone, are known from

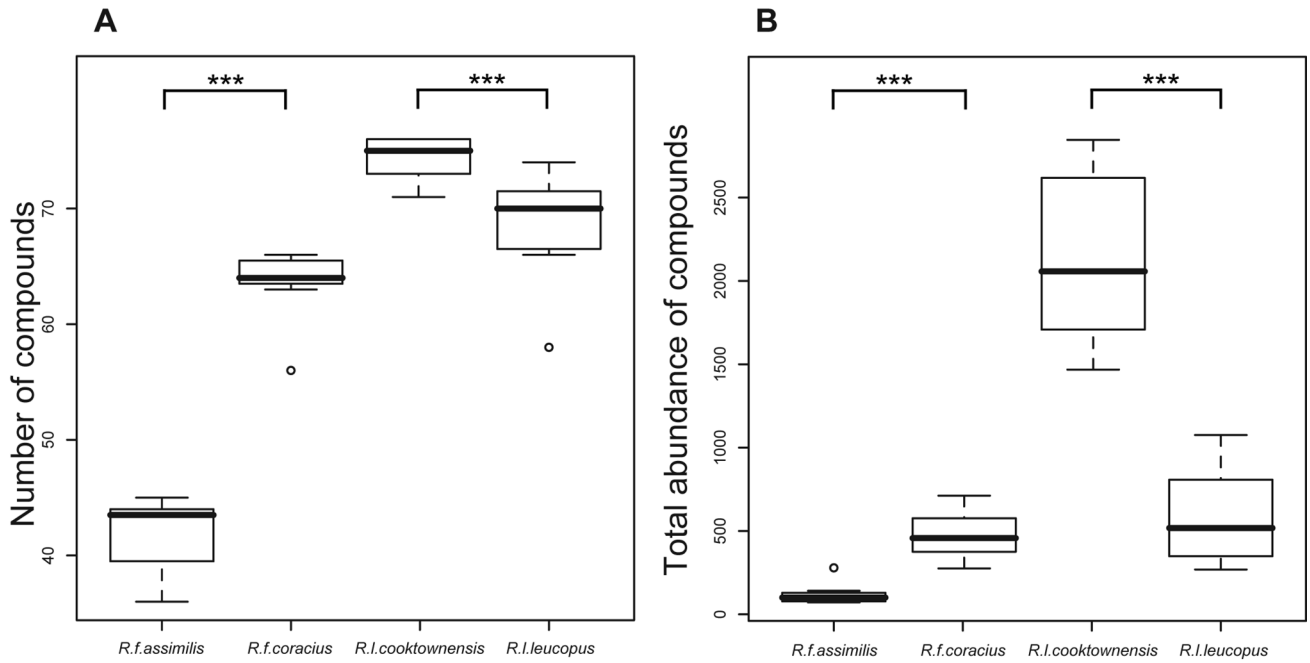

Figure 6. Variation in chemical complexity of preputial gland extracts among four subspecies of *Rattus fuscipes* and *R. leucopus* based on 80 quantitated compounds. (*A*) Box and whisker plots of the number of chemical compounds detected in each subspecies. (*B*) Box and whisker plots of the total abundance of chemical compounds detected in each subspecies. In both, asterisks above pairwise comparisons of conspecific subspecies indicate significantly higher values ($p < 0.01$) with a one-way Mann-Whitney U Test for all sympatric to allopatric comparisons.

urine of *Mus musculus* where they are involved in male effects on female estrus (Jemiolo *et al*., 1989) and activate specific vomeronasal organ neurons (Leinders-Zufall *et al*., 2000). 2-Heptanone also is known from preputial glands of *Rattus norvegicus*, where it is associated with social stress (Gutierrez-Garcia *et al*., 2006; Pohorecky *et al*., 2008). Notably, we did not detect farnesenes in *R. fuscipes* or *R. leucopus.* Farnesenes are sesquiterpenic compounds that originate in and are common components of rodent preputial glands, including in *Mus musculus* and *Rattus norvegicus*, where they signal social dominance (Harvey *et al*., 1989; Novotny *et al*., 1990; Pohorecky *et al*., 2008; Zhang *et al*., 2008). The partially identified geranyl-related sesquiterpenes observed in this study may have similar functions, replacing the farnesenes in *R. fuscipes* and *R. leucopus*. Two of these were detected only in *R. fuscipes*, but a third was detected in both *R. fuscipes* and *R. leucopus*.

Overall, our multidimensional analysis of chemical composition of preputial glands clearly separated *Rattus fuscipes* and *Rattus leucopus.* We found only a handful of compounds that are exclusive to each species, but the relative abundance of compounds was significantly different between species, suggesting that they are clearly differentiable by their chemical signatures. Several studies have demonstrated that the relative abundance of chemical compounds is often a reliable indicator of perceivable differences among sexes or species (Johansson & Jones, 2007; Zhang *et al*., 2007, 2008a; Apps, 2013). However, our total analysis considered the entire pool of 80 VOCs quantitated by GC-MS, which might not all be perceivable or relevant to signalling in *Rattus.* Thus, we also analysed a subset of thiazolines, carboxylic acids, and geranyl-related sesquiterpenes that are known to be relevant in chemical signaling in mammals (Schwende *et al*., 1986; Zhang *et al*., 2008b). This subset of 17 compounds showed an equivalent pattern, with differentiation of species by their chemical composition, suggesting that odours of *R. fuscipes* and *R. leucopus* are reliably species-specific. These results are consistent with studies in other rodents, particularly in the genus *Mus*, that find clear compositional differences among species and genetic lineages (Smadja & Butlin, 2009; Soini *et al*., 2009; Hurst *et al*., 2017).

In addition to differences among species, we uncovered substantial variation within species. First, the chemical composition among subspecies within both *R. fuscipes* and *R. leucopus* were as distinguishable as between species. One of the great challenges of taxonomy is to differentiate population-level or geographic variation (i.e. subspecies) from fixed differences among species. Indeed, we observed the highest number of fixed differences in chemical composition between subspecies of *R. fuscipes*, with 16 chemical compounds found in *Rfc* that were absent in *Rfa.* If treated as taxonomic characters, these might suggest species boundaries within *R. fuscipes* that could be positively misleading. While species and subspecies are differentiable in our multidimensional analyses of chemical composition, subspecies of the same species are not clearly closer to each other in multidimensional space (Fig. 5). This is primarily because of the divergence of *Rfa* and *Rfc* from each other, whereas *Rlc* and *Rll* are in close proximity. In addition to variation among subspecies, we also recovered considerable variation in chemical composition among individuals. This is not surprising given that chemical signals are used to identify individuals within populations and to communicate information about their gender, status, and condition (reviewed in Brennan & Kendrick, 2006; Ferrero & Liberles, 2009). In addition, many variables, not all chemical signals or indicators, contribute to the biochemical differences among individuals (Novotny *et al*., 2007, 2008; Zhang *et al*., 2008)

Many factors contribute to the biochemical differences among species and individuals (Brennan & Kendrick, 2006). A genetic basis for biochemical differences among species is evident in studies between and within species of rodents (Yamaguchi *et al*., 1981; Hurst *et al*., 2017). However, changes in species' metabolic systems also can alter their biochemical signatures (Zhang *et al*., 2007). Both *R. fuscipes* and *R. leucopus* are at the latitudinal limits of their respective ranges. Where they are sympatric, they co-occur at mid-elevations, with only *R. fuscipes* at the highest and only *R. leucopus* at the lowest elevations (Table S1, Rowe *et al*., 2020). These latitudinal and elevation differences are expected to underlie significant physiological differences between the species (Collins, 1973) that may affect their biochemical composition. In addition, ecological differences between species, including diet (Havlíček *et al*., 2019) and microbiotic community (Archie & Theis, 2011; Davis *et al*., 2013), can change species' biochemical composition. For example, ketone-related compounds, some of which we detected exclusively in *Rattus leucopus*, are produced by bacteria in the uropygial gland of the songbird species *Junco hyemalis* (Whittaker *et al*., 2019).

Despite variation among subspecies and individuals there is some evidence that preputial gland chemical composition, whatever its source, is relevant to maintaining species boundaries between *R. fuscipes* and *R. leucopus*. For one, we found a consistent pattern of increased complexity of chemical composition in both species where they are sympatric with each other compared to allopatric populations. Both species have significantly more compounds and more abundance of compounds per gram of preputial gland in sympatry than their respective allopatric conspecifics (Fig. 6). We also detected a handful of compounds that are unique to *R. leucopus* (n = 8) or *R. fuscipes* (n = 2), and found in both subspecies of each, respectively. These species-specific compounds warrant further study to test if they have any function in intraspecific communication. The two partially identified geranyl-related sesquiterpenes in *R. fuscipes* may play an important role in social interactions, similar to farnesenes in other rodents (Gutierrez-Garcia *et al*., 2006; Pohorecky *et al*., 2008). In addition, we identified ten compounds that have the greatest contributions to chemical proportions differentiating both species (*R. fuscipes* and *R. leucopus*) and sympatric subspecies (*Rfc* and *Rfa*) but with lower contributions to differences between allopatric conspecifics (*Rfc* and *Rfa*, *Rlc* and *Rll*; Table 3). One of these compounds, SBT, is an important social communication compound in *Mus musculus,* including signalling social status and for attracting females (Jemiolo *et al*., 1985; Novotny *et al*., 1985; Schwende *et al*., 1986). Its proportional contribution to chemical composition of the preputial gland is more than two times greater in *R. fuscipes* than in *R. leucopus* and is most different between sympatric subspecies *Rfc* and *Rlc* (Table 2). These patterns from sympatric congeneric species and from allopatric conspecific subspecies suggest that compositional differences in compounds could be used to identify and maintain species boundaries in wild *Rattus*.

Native Australian *Rattus* have one of the fastest rates of speciation reported for mammals but show limited morphological disparity among species (Rowe *et al*., 2011). *Rattus fuscipes* and *Rattus leucopus* occur in sympatry where they are difficult to distinguish based on external morphology (Taylor & Calaby, 1988; Lidicker & Laurance, 1991), but show no evidence of gene flow, which we confirmed here with allozymes. Our chemical analyses support the hypothesis that such phenotypically cryptic species are likely to rely on chemical cues for mating signals and to be distinguishable by the chemical composition of their primary secretory scent glands, the preputial glands. Rapid chemical evolution among closely related species may explain the rapid evolution of reproductive boundaries despite postzygotic reproductive compatibility (Higgie *et al*., 2000; Zozaya *et al*., 2019). However, three other species of Australian *Rattus* (i.e. *R. sordidus*, *R. colletti*, and *R. villosissimus*) are models for speciation via postzygotic incompatibilities caused by rapid chromosomal rearrangements (Baverstock *et al*., 1977, 1983). Notably, these three species have diverged from each other more recently than subspecies within *R. fuscipes* (Rowe *et al*., 2011). Thus, within the recent and rapid radiation of Australian *Rattus*, both premating and postmating mechanisms are likely to have evolved rapidly to maintain reproductive barriers among species. The chemical composition of preputial gland secretions from other Australian *Rattus* are entirely unknown. We would predict that chemical differences among species evolved rapidly to help them avoid incompatible matings with their closest relatives, especially in lineages that are sympatric such as *R. fuscipes* and *R. leucopus* or with strong postzygotic barriers to reproduction such as *R. colletti*, *R. sordidus,* and *R. villosissimus*. Our study highlights the rich diversity of chemical compounds in preputial glands of wild rodents and the qualitative and quantitative differences among species that warrant further examination across the tree of life.

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Special note from the first author: This manuscript and the enclosing volume is dedicated to the life and work of Dr Ken Aplin. Much like the chemical composition of *Rattus* in this study, Ken could identify and differentiate many of the most morphologically-similar vertebrates, from lizards to rats, often with the most fragmentary of material such as incisors from owl pellets. In my early career as an international postdoctoral fellow, Ken's generosity of time and knowledge allowed the success of several papers on rodents, particularly Australo-Papuan *Rattus*, that formed the foundation of my career in Australia. As an immigrant, blessed with a position at one of Australia's leading natural history collections, with a family raised in this country, and with a research program centred on the Australian continent, I am forever grateful to Ken for his early role in making that all possible for me.

Supplementary data

Table S1 and Table S2 are published separately by the authors, see Rowe *et al*., 2020.

Table S1. Sample metadata, allozyme results and Genbank Accession numbers (Rowe *et al*., 2020: table S1).
https://doi.org/10.6084/m9.figshare.13058855

Table S2. Gas chromatography-mass spectrometry results for preputial gland extractions from *Rattus fuscipes assimilis* and *R. f. coracius* (*Rfa-Rfc*) and for *R. leucopus cooktownensis* and *R. l. leucopus* (*Rlc-Rll*). Compound identity verified by a standard; S and N verified by atomic emission detection; * ion 55, ion 60, etc. means that the peak area was integrated in the post-run single ion chromatogram (SIC); TIC = total ion chromatogram. Different molecular branching types denoted "A", "B", "C". For diketones "x" denotes unknown substitution site. QMJM is the Queensland Museum specimen number (Rowe *et al*., 2020: table S2).
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