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Saltational evolution of contact sex pheromone compounds of *Callosobruchus rhodesianus* (Pic)

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Abstract *Callosobruchus rhodesianus* (Coleoptera: Chrysomelidae: Bruchinae) is a pest of stored legumes occurring throughout the Afro-tropical region. The females of these beetles produce a contact sex pheromone that elicits copulation behavior in males. To identify the contact sex pheromone compound, cuticular compounds of virgin females were collected using a filter paper method. The compounds were fractionated by acid–base partition and chromatography techniques and assayed for their ability to elicit male copulatory activity. Gas chromatography–mass spectrometry analysis of the active fraction revealed that the main active compounds were hexahydrofarnesyl acetone (6,10,14-trimethyl-2-pentadecanone) (**1**) and 2-nonadecanone (**2**), and the synergists were C27–C33 hydrocarbons (HCs), n-alkanes, and a homologous series of mono- and dimethyl branched alkanes. The main compounds (**1**) and (**2**) were substantially different from the contact sex pheromones (monoterpene dicarboxylic acids) previously identified from congeneric species. Copulatory assays using synthetic standards together with the natural HCs revealed that both ketone compounds were needed for a full male response. The present results explain the mate recognition specificity of *C. rhodesianus*, and the differences between the pheromones in the beetles may be explained by saltational evolution.

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Introduction

It is commonly accepted that efficient communication requires reciprocal adaptations in both the sender and receiver of a signal. Although evolutionary change in sexual signals such as sex pheromones is necessary for the divergence of mate recognition specificity, the necessity as a functional signal restricts the range of the change (Butlin and Ritchie 1989; Allison et al. 2008). Such a sexual signal should be under strong stabilizing selection because a slight change in the signal would lead to less than optimal mutual recognition (Symonds and Elgar 2008). Therefore, for the diversity of sexual signals, the evolution proceeds by more major saltational evolution, which generates greatly or completely different phenotypes from those possessed by the ancestor (Baker 2002).

In insect species, sex pheromones provide important signals in the reproductive communication process. Volatile sex pheromones are released to attract the other sex. Non- or less-volatile sex pheromones aid in mating discrimination and elicit courtship (Wyatt 2003). Accordingly, the speciation process is often accompanied by sex pheromone diversification (Smadja and Butlin 2009).

Callosobruchus seed beetles (Coleoptera: Chrysomelidae: Bruchinae) are serious pests of stored food and are distributed worldwide (Rees 1996, 2004). In several *Callosobruchus* species, contact sex pheromone compounds have been found to be triggers for males to attempt mating, and this stimulus is vitally important for the choice of a

mating partner by the male (Tanaka et al. 1981; Nojima et al. 2007; Shimomura et al. 2010b). In our previous work, the male mate discrimination specificities among four congeneric *Callosobruchus* seed beetles (*C. chinensis*, *C. maculatus*, *C. analis,* and *C. rhodesianus*) were clearly distinguishable. Males of both *C. chinensis* and *C. maculatus* lack the ability to discriminate in favor of conspecific mates. However, males of *C. analis* and *C. rhodesianus* discriminate in favor of their conspecific mates (Shimomura et al. 2010a). As for the four seed beetles, *C. chinensis*, *C. maculatus*, and *C. analis* are widely distributed. *C. chinensis* is present in the temperate New World, Neotropical, Afrotropical, Middle East, Indochina, Tropical Asia and temperate Old World. *C. maculatus* is distributed in the Neotropical, Afrotropical, Middle East, Indochina, Tropical Asia and temperate Old World. *C. analis* is found in the Afrotropical, Indochina and Tropical Asia, while *C. rhodesianus* is locally distributed in the Afrotropical (Tuda et al. 2005).

Because the male mate discrimination specificities are considered to be due to the effects of contact sex pheromone compounds, several works to identify the compounds have been performed; currently, those from *C. chinensis*, *C. maculatus*, and *C. analis* have been reported. Commonly, the compounds are synergistic mixtures of monoterpene dicarboxylic acids and certain hydrocarbons (Tanaka et al. 1981; Nojima et al. 2007; Shimomura et al. 2010b). Therefore, the identification of the pheromone compounds of *C. rhodesianus* has been eagerly awaited. In this study, we characterize the contact sex pheromone compounds of *C. rhodesianus* and reveal their structures, which are unexpectedly different from those of the other three beetles. The results explain how the mate discrimination specificity in *C. rhodesianus* is realized.

Materials and methods

Beetles

A laboratory-maintained colony was used in this research, which originated in South Africa and was maintained at the Yokohama Plant Protection Station, The Ministry of Agriculture, Forestry and Fisheries of Japan. *C. rhodesianus* beetles were reared on adzuki beans (*Vigna angularis*) in a dark incubator at 28 °C and ambient humidity. Shortly after emergence from the beans, adults were separated according to sex. Males were kept separately in a glass vial (15 mm in diameter, 35 mm high) and conditioned for 1 day at 27 °C and ambient humidity in a dark incubator.

Collection of cuticular compounds

Each set of 500 virgin females was kept in a glass container (90 mm in diameter, 100 mm high) for 14 days with corrugated filter paper shelters to collect cuticular compounds rubbed onto the filter paper. The filter paper shelters were collected from the containers and were cut finely and Soxhlet-extracted with ether for 24 h. The inside surfaces of the glass containers were rinsed with ether, which was then combined with the filter papers. The extracts were dried with anhydrous Na_2SO_4. After filtration, the solvent was evaporated under reduced pressure. The extracts were kept at −30 °C until use.

Purification of cuticular compounds

Crude cuticular compounds were fractionated into acidic, neutral, and basic fractions. The crude extract was dissolved in 30 ml of ether and extracted twice with 20 ml of 0.5 M HCl. The acidic aqueous layer was alkalized with 5 M NaOH and extracted twice with 20 ml of ether to recover the basic compounds, and this ether layer was washed with brine and dried with anhydrous Na_2SO_4. Acidic compounds were extracted from the original ether layer twice with 20 ml of 0.5 M NaOH. The alkaline aqueous layer was acidified with 5 M HCl and extracted twice with 20 ml of ether to recover the acidic compounds, and the ether layer was washed with brine and dried with anhydrous Na_2SO_4. The remaining ether layer was washed with brine and dried over anhydrous Na_2SO_4. After filtration, each fraction was concentrated under reduced pressure.

Column chromatography

The neutral fraction was redissolved in ether and concentrated to dryness with 700 mg of silica gel in a rotary evaporator. The silica gel was applied to the top of a prewetted 1.5 g silica gel column. The neutral compounds were eluted sequentially with 25 ml each of hexane, 5 % ether in hexane, 10 % ether in hexane, 20 % ether in hexane, 50 % ether in hexane, and pure ether.

High-performance liquid chromatography (HPLC)

The active fraction was further purified by normal-phase HPLC with an LC-10A system (Shimadzu, Kyoto, Japan) equipped with a silica gel column (Inertsil SIL-100A, 3 μm, 4.6 mm × 250 mm, GL Science, Tokyo, Japan). Compounds were eluted with 5 % ether in hexane (1 ml/min). The effluent was monitored with a photodiode array detector SPD-M10Avp (Shimadzu) and collected in 1-ml fractions.

Gas chromatography–mass spectrometry (GC-MS) and GC analysis

GC-MS analysis was carried out with a Shimadzu GC 17A instrument equipped with a DB-5 or DB-23 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) coupled to a Shimadzu QP-5000 quadrupole MS in EI mode (70 eV). Helium was used as the carrier gas at a head pressure of 100 kPa and a flow rate of 1.6 ml/min. For analysis of ketone compounds, the oven temperature was initially set at 60 °C for 3 min and was increased at a rate of 10 °C/min until 280 °C was achieved for the DB-5 column and 220 °C for the DB-23. Those temperatures were held for 10 min. The injector and interface temperatures were set at 250 and 280 °C, respectively. The active compounds were identified by comparing their mass fragmentation patterns with those of the NIST library, and the identities were confirmed by comparison with synthetic standards. For hydrocarbon analysis on the DB-5 column, the initial temperature was set at 80 °C for 3 min, increased at a rate of 12 °C/min until 320 °C was reached, and then held for 10 min. The injector and interface temperatures were both set at 320 °C.

For the estimated ratio of active contact sex pheromone compounds, GC analysis was carried out with an HP 5890 series II instrument with a flame ionization detector. The analytical condition was the same as the condition of the GC-MS analysis. The estimation of the ratio of the active contact sex pheromone compounds was performed using the peak area percentage method.

Chemicals

Hexahydrofarnesyl acetone (6,10,14-trimethylpentadecan-2-one) (**1**) was prepared to >95 % purity by a previously reported method (Sasaerila et al. 2003). 2-Nonadecanone (**2**) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Behavioral bioassay

Males in vials were removed from the incubator and acclimated to conditions in an assay room for 1 h before the copulatory assay. The assay was performed at 27 °C under lighted conditions. The tip of a glass rod (1.5 mm i.d., 100 mm length) was rounded and abraded to serve as a surrogate for the body of a female beetle. The concentration of each extract or synthetic compound was adjusted to 0.1 female equivalent (feq)/μl by dissolving ether. It should be noted that feq calculations were based on filter paper extracts rather than on female whole body extracts. Five microliters of each sample was applied to each dummy using a microsyringe. After the solvent had evaporated, the dummy was presented to the male in the vial. Copulatory activity was evaluated by observing whether males displayed their genital organ and tried to copulate within 2 min in each assay. We had conducted three sets of the behavioral bioassay; in the first experiment, we tested the crude extract, its acidic, neutral, and basic fractions as well as the recombined fractions; in the second experiment, we tested the neutral fraction purified by column chromatography and HPLC; and in the third experiment, we tested synthetic standards with the neutral hexane fraction for their ability to elicit copulatory behavior in males. We included a control (ether) in each of the experiments described above. For all samples and controls, 20 replicates were conducted using a new male and a newly prepared surrogate for each replicate.

Statistical analysis

The numbers of males that extruded their genital organs in response to the sample were compared to the numbers responding to a surrogate treated with only the solvent using Fisher's exact probability test. Multiple comparisons for male copulatory activity were performed by Fisher's exact probability test followed by a series of sequential Bonferroni corrections (Rice 1989). Both the corrected and uncorrected P values are presented in the text in the "Results" section, and uncorrected P values are given in parentheses. All statistical analyses were performed using the excel statistics software Ekuseru-Toukei 2010 program (Social Survey Research Information Co., Ltd, Tokyo, Japan).

Results

The cuticular compounds of female *C. rhodesianus* were collected from approximately 5400 females. The yield of the crude extract was 353.2 mg, and the extract (0.5 feq, applied on a glass rod) elicited 95 % strong copulatory activity from *C. rhodesianus* males [Fisher's exact test, corrected $P < 0.001$ (uncorrected $P < 0.001$); Fig. 1a]. The crude extract was partitioned into acidic, neutral, and basic fractions. Only the neutral fraction elicited strong copulatory activity [90 % response; Fisher's exact test, $P < 0.001$ ($P < 0.001$); Fig. 1a]. The neutral fraction was separated by silica gel column chromatography into six fractions. Although each fraction was assayed, only the 5 % ether fraction (Fr. 2) [20 % response; Fisher's exact test, $P = 0.636$ ($P = 0.106$); Fig. 1b] and the 10 % ether fraction (Fr. 3) [5 % response; Fisher's exact test, $P = 1$ ($P = 1$); Fig. 1b] elicited any copulatory responses (albeit not at significant levels). As only one male showed a copulatory response toward Fr. 3, only Fr. 2 was further

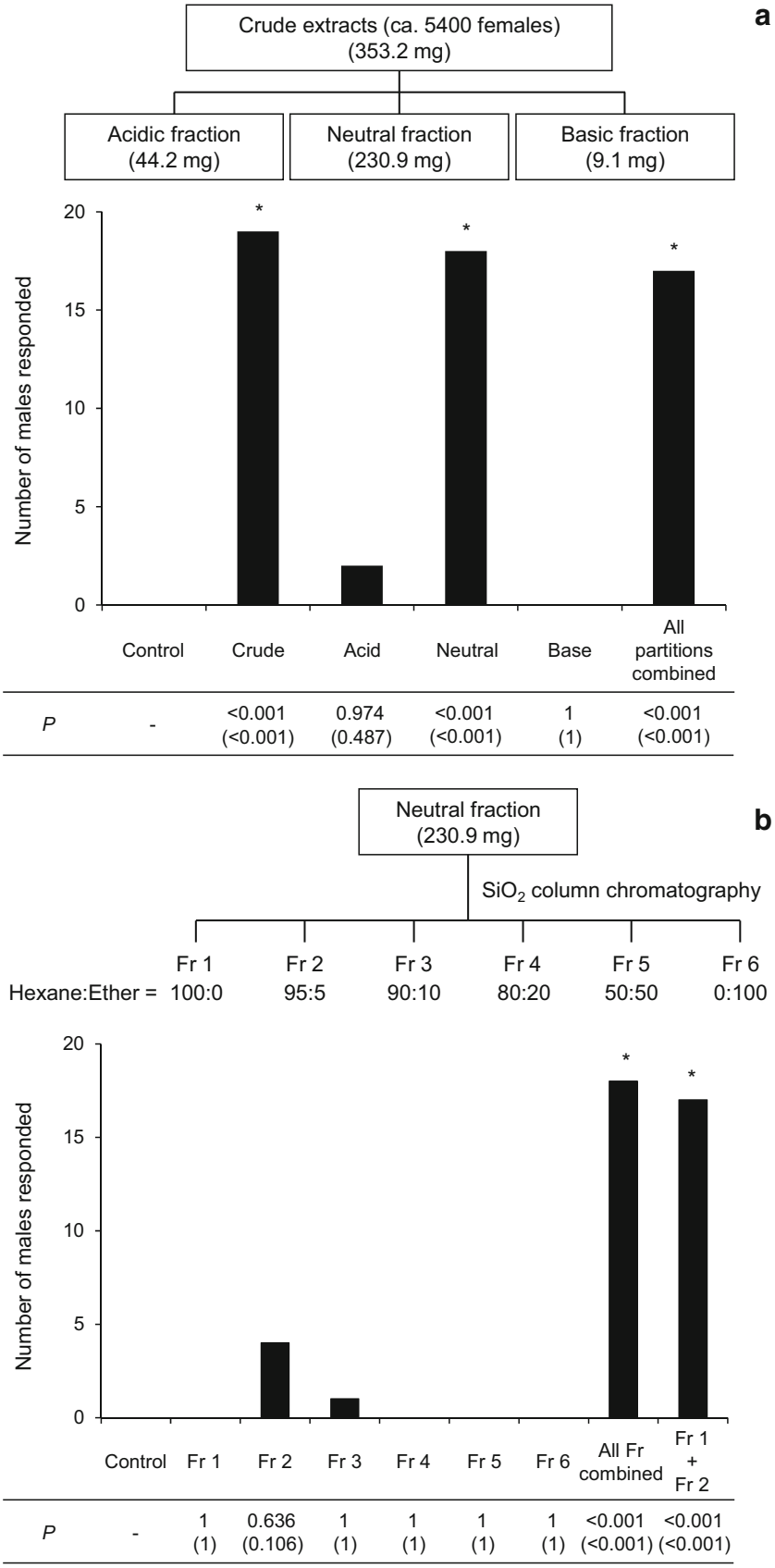

Fig. 1 Diagram of the fractionation procedure and male *Callosobruchus rhodesianus* copulatory responses to glass rod dummies with each applied sample. **a** Crude extract collected from female *C. rhodesianus* by the filter paper method and separated into acidic, neutral, and basic portions. **b** Active neutral portion further resolved by silica gel column chromatography into six fractions (Fr. 1–6). Twenty males were tested for each sample. All samples were tested at 0.5 female equivalents. *Asterisks* above *bars* indicate significant differences between the test sample and the control (solvent applied) (Fisher's exact probability test followed by sequential Bonferroni corrections, $P < 0.05$). The list of values shown below each sample represents corrected and uncorrected P values in the *top* and *bottom* in parentheses, respectively

investigated. When the hexane fraction (Fr. 1) was combined with Fr. 2, the copulatory activity was restored [85 % response; Fisher's exact test, $P < 0.001$ ($P < 0.001$); Fig. 1b]. This was almost the same activity as that obtained when all fractions were combined (Fig. 1b).

The 5 % ether fraction was further purified by normal-phase HPLC, and copulatory behavior was elicited when the 13–14 min [80 % response; Fisher's exact test, $P < 0.001$ ($P < 0.001$)] and 14–15 min [70 % response; Fisher's exact test, $P < 0.001$ ($P < 0.001$)] fractions were combined with the hexane fraction. The two active fractions were combined and subjected to GC-MS analysis. GC-MS analysis of the active HPLC fraction revealed the presence of two compounds, compound **1** and **2** (Fig. 2a). Comparison of their mass fragmentation patterns with those of compounds in the NIST library suggested that the isolated compounds were hexahydrofarnesyl acetone (6,10,14-trimethyl-2-pentadecanone) (**1**) (Schulz et al. 2011) (Fig. 2b) and 2-nonadecanone (**2**) (Fig. 2c). The suggestions were confirmed by comparison with synthetic standards. As assessed by GC, the estimated ratio of compound **1** to compound **2** was approximately 1:5.3. Because the yield of the active fraction was 270 µg, the contents per female equivalent collected by filter paper were approximately 8 and 42 ng for compounds **1** and **2,** respectively.

GC-MS analysis of the hexane fraction revealed a number of hydrocarbons (HCs). Upon comparison of MS fragmentation patterns and retention indices with reported values (Baker and Nelson 1981; Howard 2001; Geiselhardt et al. 2009), the HCs were tentatively identified as C27–C33 HCs, n-alkanes, and a homologous series of mono- and di-methyl branched alkanes (Fig. 3). The proportion of HCs to the crude extract was approximately 50 %.

To confirm that the identified ketone compounds are the biologically active contact sex pheromones, we performed copulatory assays using synthetic standards combined with HCs from the hexane fraction (Fr. 1). As expected, the

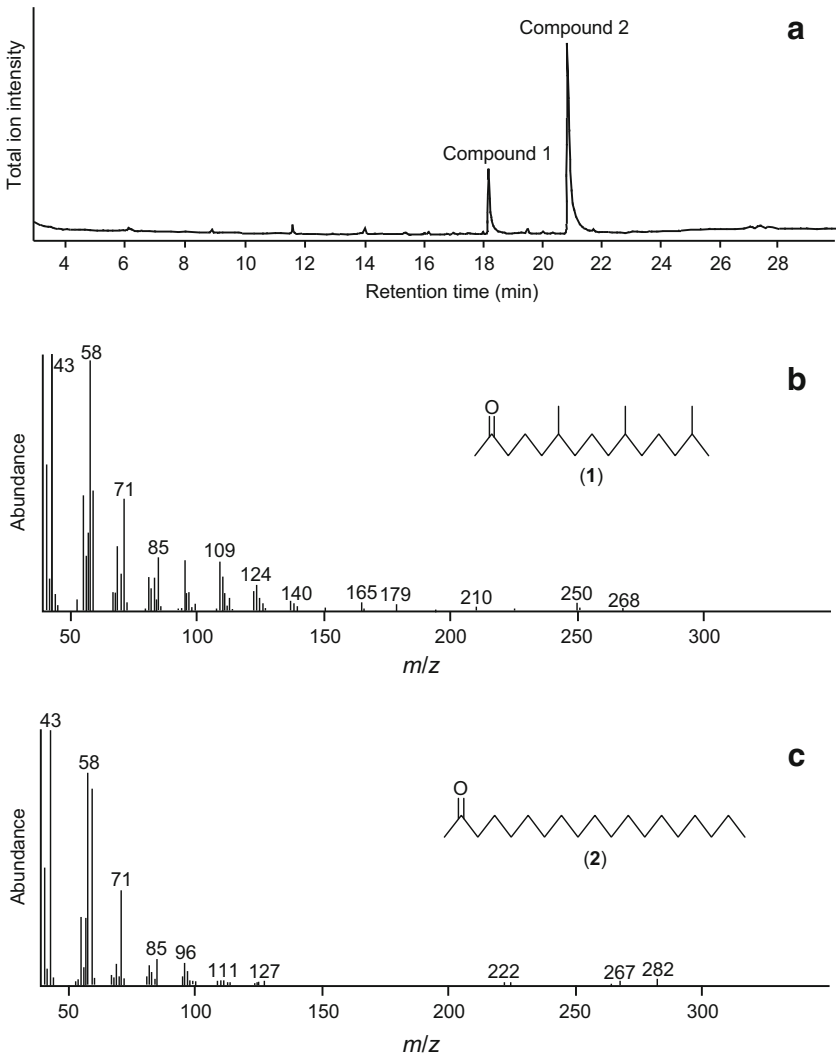

Fig. 2 GC-MS spectra of the active fraction from the original neutral fraction. **a** Total ion chromatogram of the active fraction. **b** EI-mass spectra of compound **1** identified as natural hexahydrofarnesyl acetone (6,10,14-trimethyl-2-pentadecanone). **c** Natural compound **2** identified as 2-nonadecanone

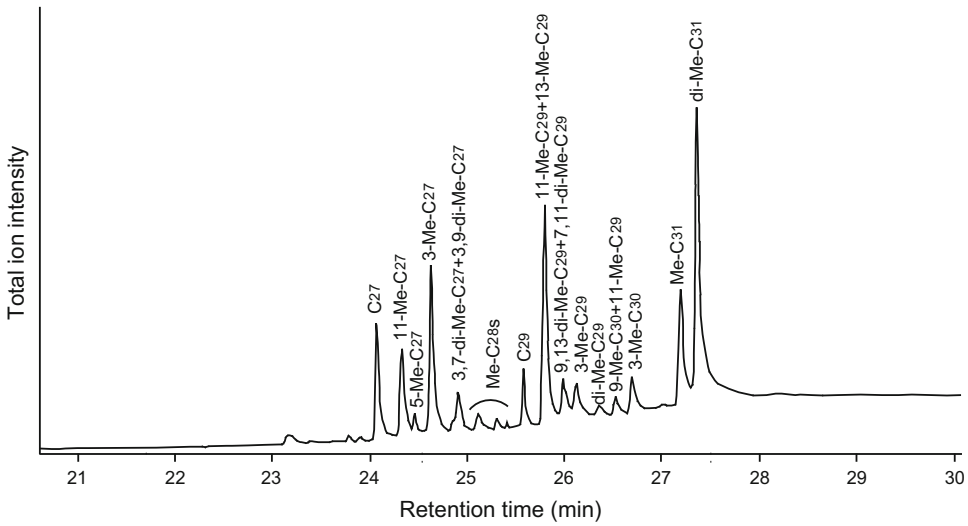

Fig. 3 Total ion chromatogram of the hydrocarbon fraction of female cuticular compounds of *Callosobruchus rhodesianus*

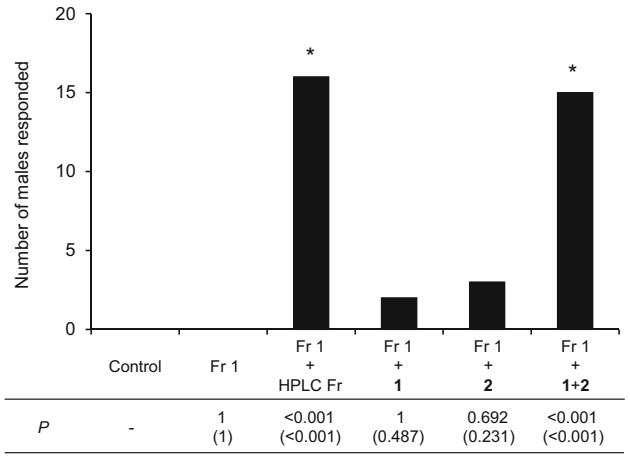

Fig. 4 Copulatory responses of male *Callosobruchus rhodesianus* to female dummies to which were applied test molecules. Fr 1 indicates natural cuticular hydrocarbons that had been eluted from silica by 100 % hexane. HPLC Fr indicates the active fraction of the HPLC eluate. Synthetic compounds, hexahydrofarnesyl acetone (**1**) and 2-nonadecanone (**2**), are indicated by their numerals. Twenty males were tested in each trial. *Asterisks* above *bars* indicate significant differences between the test fraction and the control (solvent applied) (Fisher's exact probability test followed by sequential Bonferroni corrections, $P < 0.05$). The list of values shown below each sample represents corrected and uncorrected *P* values in the *top* and *bottom* in parentheses, respectively

combination of **1** and **2** with HCs exhibited significant copulatory activity from male *C. rhodesianus* (75 % response; Fisher's exact test, $P < 0.001$ ($P < 0.001$); Fig. 4). The response was almost the same as that elicited by the active HPLC fraction combined with HCs. When applied individually rather than in combination, neither **1** nor **2** alone with HCs exhibited significant copulatory activity from male *C. rhodesianus* (Fig. 4).

Discussion

In the course of studies on contact sex pheromones of seed beetles, we investigated the copulatory activity of male *C. rhodesianus* beetles in response to cuticular compounds collected from virgin females of *C. rhodesianus*. The result was in accordance with the male copulatory activity in response to freeze-killed females of *C. rhodesianus* (Shimomura et al. 2010a).

Because contact sex pheromone compounds previously identified from three congeneric seed beetles are dicarboxylic acids with hydrocarbons as synergists (Tanaka et al. 1981; Nojima et al. 2007; Shimomura et al. 2010b), we first expected that the contact sex pheromone compounds would be similar compounds. Therefore, crude extracts were fractionated into acidic, neutral, and basic fractions. To our surprise, the case of *C. rhodesianus* was quite different, and only the neutral fraction elicited copulatory activity from male *C. rhodesianus* (Fig. 1a). From that result, we hypothesized that male *C. rhodesianus* recognized a different class of female cuticular compounds as the trigger for copulatory activity. To identify the contact sex pheromone compounds, the crude extract was fractionated by column chromatography and HPLC, and fractions were assayed for their ability to elicit copulatory activity from male beetles.

The active neutral fraction was purified by column chromatography. Male *C. rhodesianus* beetles exhibited copulatory activity in response to a combination of the hexane and 5 % ether fractions (Fig. 1b). The 5 % ether fraction was further fractionated using HPLC, and GC-MS analysis of the active HPLC fraction revealed the presence of the two active ketone compounds, hexahydrofarnesyl acetone (**1**) and 2-nonadecanone (**2**) (Fig. 2). Although

individual applications of either synthetic **1** or **2** (together with the HCs) elicited no significant copulatory activity from male *C. rhodesianus* beetles, the combined application of both of those synthetic standards of compounds **1** and **2** (together with the natural HCs) elicited significant copulatory activity from male *C. rhodesianus*, indicating that both ketone compounds are needed for a full biological response (Fig. 4).

Herein, we clarified that the main contact sex pheromone compounds of *C. rhodesianus* are **1** and **2**, and at least one HC acts as a synergist. The three congeneric seed beetles, *C. chinensis*, *C. maculatus*, and *C. analis*, use contact sex pheromone compounds that are closely related to each other within that set of three species. The main compounds are monoterpene dicarboxylic acids: (*E*)-3,7-Dimethyl-2-octene-1,8-dioic acid for *C. chinensis*; 2,6-Dimethyloctane-1,8-dioic acid for *C. maculatus*; and (2*S*,6*R*)-2,6-Dimethyloctane-1,8-dioic acid and (*S*,*E*)-3,7-dimethyl-2-octene-1,8-dioic acid for *C. analis* (Tanaka et al. 1981; Nojima et al. 2007; Shimomura et al. 2010b). Among the three seed beetles, *C. chinensis* and *C. maculatus* lack stereospecificity toward their dicarboxylic acids, but *C. analis* exhibits stereospecificity toward both of its dicarboxylic acids (Mori et al. 1983; Yajima et al. 2006, 2007; Shimomura et al. 2010b). The stereochemistry–activity relationship is quite related to the fine-tuned mate recognition specificity of *C. analis*. On the other hand, the present results indicate that the fine-tuned mate recognition specificity of *C. rhodesianus* is achieved by the substantial structural difference of the main contact sex pheromone compounds away from the molecules of the congeneric seed beetles.

In insect sexual communication, sex pheromones are widely used and the chemical structures and blends of the sex pheromones have extraordinary diversity. Generally, sex pheromones are under strong stabilizing selection because an aberrant change of signal or receptor would hamper mating (Groot et al. 2006). An evolutionary change of signaling compounds would be made resistant to further change because the necessity of a functional signal restricts the range of possibilities (Butlin and Ritchie 1989). There are two distinct ways in which pheromone compounds can evolve (reviewed in Symonds and Elgar 2008). The first involves a gradual process of small changes in compounds or a change in the relative proportions of the compounds over evolutionary time, such that closely related species share closely related or identical compounds (Roelofs and Brown 1982). In the second process, major changes induce saltational shifts, which generate a new phenotype that is greatly or completely different from the antecedent (Baker 2002). This mode of evolution results in highly dissimilar pheromone compounds and reduces or prevents interspecific responses on sibling species (Symonds and Elgar 2008). It has been proposed that directional selection through sexual communication interference between sympatric species that use similar sex pheromone compounds counteracts such stabilizing selection against pheromone evolution (Gries et al. 2001; McElfresh and Millar 2001; Groot et al. 2006).

In congeneric seed beetles, there are three reports of contact sex pheromone compounds commonly using dicarboxylic acids and hydrocarbons as the synergism from *C. chinensis*, *C. maculatus*, and *C. analis* (Tanaka et al. 1981; Nojima et al. 2007; Shimomura et al. 2010b). Meanwhile, in the present study, *C. rhodesianus* uses two ketone compounds and at least one HC as synergists, which are quite different from previously identified contact sex pheromone compounds. These results suggest that saltational change has arisen in the contact sex pheromone of *C. rhodesianus. C. rhodesianus* is found mainly in southern Africa and is also distributed around the equator, where it has been sporadically reported (Southgate 1979; Giga and Smith 1983). In this narrow distribution, *C. rhodesianus* and *C. maculatus* have been sympatrically observed in Togo and Benin (Tchassanti and Glitho 1995). Alternatively, a molecular phylogenic study using mitochondrial DNA showed that *C. maculatus* is a sister species to a group containing *C. rhodesianus* (Tuda et al. 2006). Furthermore, in a laboratory competition assay of these species, the number of eggs laid by *C. rhodesianus* was greatly reduced by the presence of *C. maculatus* (Giga and Canhão 1997). Combined with our results in this study, the sexual communication interference could cause directional selection, which would lead to the saltational evolution of pheromone compounds between *C. rhodesianus* and *C. maculatus*.

Symonds and Elgar (2004) reported an investigation on the relationships between chemical compounds of aggregation pheromones of 34 bark beetles from two genera, *Dendrocctonus* and *Ips*, as well as the phylogenetic traits of the bark beetles. Within each genus, the pheromone compounds of closely related species were more different from distantly related species, suggesting that pheromone evolution in bark beetles is characterized by large saltational shifts (Symonds and Elgar 2004). In a recent report, Weiss et al. (2015) reported that three sympatric *Leptopilina* parasitoid wasp species made a distinction in the species specific mate recognition pheromones. Although cuticular hydrocarbons and iridoids have been used as the mate recognition pheromones in all three species, the degree of contribution as the pheromone was quite different; *L. heterotoma* uses the iridoids as the main pheromone; in *L. boulardi*, cuticular hydrocarbons are as important as the iridoids; in the case of *L. victoriae*, the cuticular hydrocarbons are sufficient to elicit a full behavioral response, indicating the possibility of saltational evolution (Weiss

et al. 2015). In light of the present results, together with related reports, saltational shifts of the sexual communication system in congeneric species may be an important factor for speciation.

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