Chemoecology (2020) 30:49–57
https://doi.org/10.1007/s00049-019-00297-x

CHEMOECOLOGY

ORIGINAL ARTICLE

# Does the orchid *Luisia teres* attract its male chafer pollinators (Scarabaeidae: *Protaetia pryeri pryeri*) by sexual deception?

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Received: 18 November 2019 / Accepted: 13 December 2019 / Published online: 1 January 2020


## Abstract

The epiphytic orchid *Luisia teres* (Asparagales: Orchidaceae) releases floral scent that attracts males of the cupreous polished chafer *Protaetia pryeri pryeri* (Coleoptera: Scarabaeidae) for pollination. We analyzed this floral scent to identify the attractant(s). When various flower parts were extracted with diethyl ether and assayed, male chafers were attracted to the extract of petals but not to that of sepals or lips. The petal extracts were chromatographed on silica gel column and the activity was found in ether and methanol fractions. Gas chromatography/mass spectrometry (GC/MS) analyses and subsequent NMR analyses revealed that both active fractions contained 2,3-dihydroxypropyl isovalerate (2,3-DHPiV) as a major compound. Natural 2,3-DHPiV showed identical retention time to that of synthetic (*R*)-enantiomer in subsequent HPLC analyses using a chiral column, while (*S*)-enantiomer was not detected. The amount of this compound was ca. 5 µg/flower. In field tests performed in Okinawa, Japan, males of *P. pryeri pryeri* were observed to fly around and land on a cotton ball impregnated with synthetic (*R*)-2,3-DHPiV, but no male was attracted to the (*S*)-enantiomer or the racemic mixture. Thus, the chemical attractant released from the flower was identified to be (*R*)-2,3-DHPiV. 2,3-DHPiV was also found in a volatile sample collected from the virgin females by GC/MS analyses using achiral column, while this compound did not occur in males. Because of the difficulty in HPLC detection of a small amount of 2,3-DHPiV, the chirality remains to be determined. Nevertheless, these results strongly suggest that the flowers of *L. teres* release the same compound as females of its pollinator *P. pryeri pryeri,* indicating that the flower of this orchid chemically mimics the female sex pheromone.

**Keywords** Sexual deception · Orchid · Floral scent · Chafer · Sex pheromone

## Introduction

Approximately 25,000 species of orchids have food-deceptive flowers and another 400 species are sexually deceptive (Cozzolino and Wildmer 2005; Nilsson 1992). Deceptive orchid flowers attract pollinators by visual and/or olfactory cues but do not offer any reward such as nectar or pollen (Bergström 1978; Schiestl et al. 1999). Deceptive, rewardless orchid flowers often exhibit higher pollinator specificity, resulting in efficient pollen transfer in a single pollinator visit (Dafni 1984). Sexual deception is an extreme form of pollinator specialization in which an orchid attracts pollinators with fraudulent sex signals (Gaskett 2011). Some of these orchids are named "bee orchids" because they have flowers of bee-like appearance that attract specific bee species, and the bees show pseudocopulatory behavior on the flower (Kullenberg and Bergstöm 1976). Endress (1994) predicted that pseudocopulation plays a role in many unstudied

Handling Editor: Marko Rohlfs.

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tropical species, such as the genera *Luisia, Haraella* and *Lepanthes* (Asparagales: Orchidaceae). An epiphytic orchid *Luisia teres* (Thunberg) Blume is distributed in subtropical and temperate regions, Okinawa and Ogasawara Iss., Kyushu, Shikoku and Honshu, Japan, and Sichuan, China (Seidenfaden 1971).

Males of the cupreous polished chafer *Protaetia pryeri pryeri* (Janson) (Coleoptera: Scarabaeidae) have been observed to fly toward, hover around, and land on flowers of *L. teres*, whereas the females were not attracted (Arakaki et al. 2016). On a flower, a male chafer holds the lip with his legs, and then inserts his head deep into the base of the flower column, where pollinia sticks onto his frons. Subsequently, some males of *P. pryeri pryeri* show pseudocopulation on the flower lips by elongating the abdomen downward and extruding the copulatory organ. When a chafer carrying pollinia visits another flower, the pollina are deposited on the flower stigma. This chafer is thus considered to be an effective pollinator for this orchid. Arakaki et al. (2016) observed that males were also attracted to orchid flowers covered with a piece of cloth that blocked their visibility, as well as to a solvent extract of flowers, which led us to hypothesize that males used olfactory cue released from the orchid flower.

The purpose of our study is to identify the chemical compound(s) released from the flower of *L. teres* that attracts males of *P. pryeri pryeri*. We also intended to clarify the possibility whether this orchid chemically mimics sex pheromone of *P. pryeri pryeri*.

## Materials and methods

### Plant

Plants of *Luisia teres* were collected from epiphytic colonies on dead pine trees in the northern part of Okinawa Island, Japan in May 2005 and May 2012. The plants were planted on tree fern boards, and grown on tree branches or in a greenhouse until use, at the Okinawa Prefectural Agricultural Research Center, Itoman, Okinawa, Japan.

### Extraction of flower

To obtain samples for bioassays, four orchid flowers from a single or different individuals were soaked together in ca. 1 ml diethyl ether in a glass vial (1.6 cm diameter × 6.0 cm height) for ca. 4 h and the ether layer was stored in a refrigerator (0–5 °C) until use. Sepals, petals and lips separated from another four flowers were separately extracted with ca. 1 ml diethyl ether in the same manner as above. The extracts were stored at − 28 °C until use.

To obtain samples for chemical analyses, petals were removed from 153 flowers and extracted with diethyl ether (13–35 flowers/5 ml ether) for 24 h three times with the same amount of the same solvent. The extracts were pooled, dried over anhydrous $Na_2SO_4$, and stored at − 28 °C until use.

### Insects

Adults of *P. pryeri pryeri* were collected at Ozato-jôshikôen Park, Nanjo, Okinawa, in July 2016. Pealed bananas put in mesh bags were hung on a branch of a tree at ca. 1.5 m above the ground, and the chafers attracted and perched on them were collected with an insect net or by hand. Eighteen females and 15 males were reared on jelly food for insects ("Kontyu Jelly", Marukan Inc., Osaka) in a plastic container (35.5 × 20.5 × 21.3 cm height) filled with moist humus to a depth of ca. 10 cm. They were allowed to mate and lay eggs in the humus. After 1 month, ca. 200 larvae were individually transferred from the container to plastic cups (11.0 cm diameter × 10.0 cm height). They were reared with moist humus until adult emergence. Newly emerged female adults were individually housed in plastic cups (8.0 cm diameter × 5.3 cm height) with tissue paper and jelly food to ensure that they stayed virgin. Male adults were reared in groups of ten individuals in separate plastic containers and were supplied with food and humus.

### Volatile collection in a glass beaker

Virgin females of *P. pryeri pryeri* were individually confined in net cages made of stainless wire (5.0 cm diameter × 5.9 cm height). Each cage housing an insect was put in a 300-ml glass beaker from 11:00 to 16:00 during which calling behavior was frequently observed (N. Arakaki, unpublished observations). The upper opening of the beaker was closed with aluminum foil. The beaker was then placed upside down to keep the insect from direct contact with the glass surface. After the calling time, the insects and net cages were removed from the beakers. To obtain volatile samples adsorbed on glass surface, the wall and bottom of the beaker were rinsed with 5 ml ether. Solvent rinses from 49 and 60 females for 2 days (total: 109 female equivalents) were obtained. Volatile samples from 20 males for 3 days were also obtained in the same manner as above. The rinses were pooled separately for the sexes.

### Silica gel column chromatography

The solvent was removed from the crude extract of orchid petals from 153 flowers at below 20 °C and to the residue was added 0.5 ml hexane. The hexane solution was applied onto a silica gel (5.0 g) column and successively eluted with 25 ml each of hexane, 5% and 50% ether in hexane, ether, methanol, and another 25 ml methanol. Each fraction was

concentrated to 1.0 ml under reduced pressure and at below 20 °C and stored at − 28 °C until use.

## Behavioral assay

For examining attractiveness of extracts from whole or parts of flower of *E. teres*, each sample containing four flower-equivalents (4 FE) of the crude extracts was impregnated as an ether solution onto a cotton ball, which was a wad of absorbent cotton wrapped with black polyester cloth and tied with plastic-coated wire to form a ball of 1.5 cm diameter, according to the manner as described previously (Arakaki et al. 2016). The cotton balls impregnated with the samples were attached on leaf stalks (= rachises) of a fern *Nephrolepis biserrata* (Sw.) Schott (Pteridopsida: Oleandraceae) using plastic-coated wire and were placed 1–2 m apart at 20–30 cm above the ground at the Urasoe-dai-kôen Park, Urasoe, Okinawa. Feral chafers of *P. pryeri pryeri* that directly touched or landed on the cotton balls, or visited them by walking after landing in the vicinity were recorded as "landed". To minimize possible positional effects on the results, the cotton balls were rearranged in positions every 1 h. Two sets of treatments were examined three times, resulting in six replications. Tests were conducted between 10:00 and 15:00 on 30 June and 1 July, 2017. Some of the chafers that landed were collected to determine the sex.

Attractiveness of the fractions obtained from column chromatography of the extract of petals was examined in the same manner as above except for the following. Each four flower-equivalents of fractions were diluted to ca. 1 ml with the same solvent and the solutions were applied onto cotton balls. The cotton balls were rearranged every 30 min. Tests had two sets and repeated four times, resulting in eight replications, conducted on 30 June 2017.

Activities of the synthetic compounds were also examined. (1) To examine attractiveness of the enantiomers of 2,3-dihydroxypropyl isovalerate (2,3-DHPiV), 20 µg of (*R*)- and (*S*)-2,3-DHPiV and 20 µg and 40 µg of the racemic mixture were separately applied onto cotton balls. The cotton balls were rearranged every 2 h because of low population. Tests were conducted on 24–25 June 2017, which had two sets and repeated four times (eight replications). (2) Subsequently, to examine the effect of (*S*)-enantiomer on attractiveness of (*R*)-2,3-DHPiV, 20 µg of (*R*)-2,3-DHPiV or its blends with the (*S*)-enantiomer at different ratios were separately applied onto cotton balls as 1 ml methanol solution, and the cotton balls were rearranged every 2 h. Tests with single set were repeated eight times from 13 to 15 July 2018. In this test and the next test, frequency of hovering by males within ca. 10 cm from the cotton ball was also counted in addition to "landing".

Attractiveness of (*R*)-2,3-DHPiV was also compared with the extracts of whole flower or petals. Synthetic (*R*)-2,3-DHPiV (20 µg) was applied as 1 ml methanol solution onto cotton balls. Extracts from four whole flowers, and those from petals removed from four flowers of a single or different individuals were applied as ca. 1 ml diethyl ether solutions. Tests had four sets and were repeated twice in resulting eight replications. Tests were conducted on 27 June 2019.

In every test, a cotton ball treated with 1 ml pure solvent (ether or methanol) was added as a negative control. Responses of males were recorded after the solvent completely evaporated (5 min for ether or 15 min for methanol).

For statistical analyses, data ($X$) were transformed to $(X+0.5)^{1/2}$ and submitted to analysis of variance. The means were subsequently ranked by paired $t$ test and significant differences were determined using Bonferroni's correction (Sokal and Rohlf 2012). In the figures, the means accompanied by the same letter are not significantly different at the 5% level.

## Gas chromatography (GC)

GC analyses were conducted using the Agilent 6890 GC equipped with split/splitless injector, an HP-INNO-Wax fused silica column (30 m × 0.25 mm inner diameter (ID) × 0.25 µm film thickness), Agilent Technologies, Santa Clara, CA, USA), and a flame ionization detector (FID; 240 °C). Samples were injected at splitless mode (1 min). Helium was used as the carrier gas at a constant flow mode of 1.0 ml/min. The column oven temperature was kept at 50 °C for 1 min, programmed at 10 °C/min to 240 °C, and held at the final temperature for 6 min. Chiral column used was an Inertcap Chramix fused silica column (30 m × 0.25 mm ID × 0.25 µm film thickness, GL Sciences Inc., Tokyo) and oven temperature was kept at 50 °C for 1 min, programmed at 3 °C/min to 180 °C, and kept at the final temperature for 5 min.

## Gas chromatography–mass spectrometry (GC/MS)

GC/MS analyses were conducted with an Agilent 6890N Network GC System combined with an Agilent 5975 inert XL mass selective detector at EI mode (70 eV). The GC was equipped with an HP-5MS fused silica column of the same dimensions as above. Injections were made through an on-column injector, which was controlled at the oven temperature plus 3 °C. The oven temperature program was 50 °C (1 min)–10 °C/min–280 °C (10 min). Helium was used at a constant flow mode of 1.0 ml/min.

For exact mass measurement, GC/MS analyses were conducted with an Agilent 7890A GC System interfaced to a JMS-T100GC time-of-flight mass spectrometer (GC/TOF–MS, JEOL, Tokyo, Japan) in EI mode with 70 eV at 200 °C. Injection was set for splitless mode at 200 °C for

1 min. The GC was equipped with the same HP-5MS column. Helium was used at constant flow mode at 1.1 ml/min. The oven temperature program was 50 °C (1 min)–10 °C/min–280 °C (10 min). To determine exact mass values, the mass spectra were measured with an internal standard, perfluorokerosene (Tokyo Chemical Industry Co., Ltd., Tokyo Japan).

For the calculation of Kovát index (KI, Kováts 1965), straight-chain alkanes with even numbers of carbon atoms from $C_{12}$ to $C_{28}$ were added to the samples before being injected into GC or GC/MS.

### High-performance liquid chromatography (HPLC)

HPLC was performed with an LC-10Ai Shimadzu Chromatographic System equipped with SPD-20A UV–Vis detector. Chromatographic separation was achieved on a CHIRALPAK®IC column (4.6 mm ID × 25 cm, Daicel Corporation, Osaka, Japan). Solvent was 20% 2-propanol in hexane flowed at 1.0 ml/min. For separation of the two enantiomers, samples were injected as 100 µl of chloroform solution using an SIL-10AL auto-injector and detected at 210 nm. Although baseline separation of synthetic (*S*)- and (*R*)-enantiomers of 2,3-DHPiV was achieved at $t_R = 8.15$ min and $t_R = 9.25$ min, respectively, the HPLC peak(s) of natural 2,3-DHPiV was hidden by those of UV-active impurities. Therefore, HPLC eluents were fractionated from 7.50 to 10.0 min at 0.25 min intervals, and subsequently 2,3-DHPiV was detected using GC equipped with the achiral column (HP-INNOWax).

### NMR analyses

$^{1}H$, $^{13}C$ and HMBC spectra of the natural compound were measured at 298 K in $CDCl_3$, using a Bruker AVANCE III HD 800 spectrometer (Bruker BioSpin, Karlsruhe, Germany) operating at 800.23 MHz equipped with a TCI ($-^{1}H/^{13}C$-{$^{15}N$}) – CryoProbe™. $^{1}H$ and $^{13}C$ chemical shifts were calibrated by the solvent residual $\delta_H$ 7.26 and solvent $\delta_C$ 77.0 signals, respectively.

### Chemicals

Racemic 2,3-dihydroxypropyl isovalerate (2,3-DHPiV) was prepared from glycerol (1,2,3-propantriol, purity > 97%, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and isovaleric anhydride in triethylamine and isolated by column chromatography on silica gel (racemic mixture, yield 37%). (*R*)- and (*S*)-enantiomers of 2,3-DHPiV were prepared from (*S*)-(+)- and (*R*)-(−)-2,2-dimethyl-1,3-dioxolane-4-methanol, respectively (98–99%ee, Sigma-Aldrich, Missouri, USA). These two enantiomers were separately immersed with isovaleric chloride (Wako Pure Chemical Industries, Osaka, Japan) in triethylamine to produce (*R*)- and (*S*)-isovalerate, respectively. Enantiomers of DHPiV were subsequently hydrolyzed by treatment with phosphomolybdic acid supported on silica gel ($H_3PMo_{12}O_{40}/SiO_2$) (Yadav et al. 2005) and subsequent purification on $SiO_2$ column chromatography. Yields of (*R*)- and (*S*)-enantiomers were 52% and 56% from the corresponding isopropylidene alcohols, respectively.

Both the enantiomers obtained were partially racemized during hydrolysis; enantiomeric purities reduced to ca. 93% ee. For obtaining pure (*R*)- and (*S*)-enantiomers, each crude enantiomer was injected into the chiral HPLC and fractions from 7.50 to 8.50 min ((*S*)-enantiomer) and from 8.50 to 9.50 min ((*R*)-enantiomer) were collected. Opposite enantiomer was not detected in the purified samples of either (*R*)- or (*S*)-enantiomer by subsequent chiral HPLC analyses.

Hexane, diethyl ether and methanol had grades for residual analysis of pesticide and polychlorinated biphenyl, which were purchased from the Wako Pure Chemical Industries. The other solvents for syntheses were also purchased from the same company. Those solvents were used without further purification.

## Results

### Attraction of males to floral extracts

Male adults of *P. pryeri pryeri* were observed to land on the balls treated with whole flower extracts in field assays performed on Okinawa Island (Fig. 1), which was consistent with previous observations (Arakaki et al. 2016). When extracts of sepals, petals and lips of the orchid flowers were

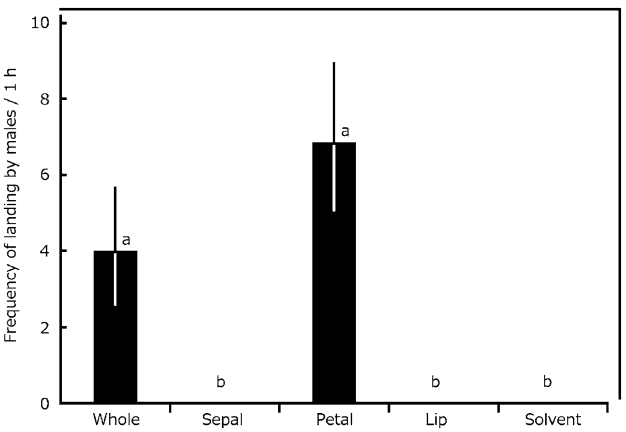



**Fig. 1** Landing by males of *Protaetia pryeri pryeri* on cotton balls treated with extracts from whole or parts of four flowers of *Luisia teres* (mean ± SE, $N = 6$). Urasoe, Okinawa, Japan, 30 June and 1 July, 2017. Means accompanied by the same letter are not significantly different ($p < 0.05$)

separately presented, males landed on the balls treated with extracts of petals but not on those of sepals or lips (Fig. 1), indicating that the attractive cues were contained in the extracts of petals of the orchid flowers.

All 60 chafers that landed were determined to be male.

## Isolation and chemical analysis

Six fractions eluted from $SiO_2$ column chromatography of the ether extract of petals of *L. teres* flowers, hexane, 5%-ether/hexane, 50%-ether/hexane, 100%-ether, and two methanol fractions were applied onto cotton balls and placed in the field, Okinawa. Male chafers were attracted to the ether and the first methanol fractions, but no male was attracted to the other fractions (Fig. 2).

GC/MS analyses of the first methanol fraction showed almost a single GC peak at $t_R = 10.83$ min (Compound A; $KI = 1334$/HP-5MS; Fig. 3a), and this compound was also found in the ether fraction. Fragment ions at *m/z* 103, 85 and 57 suggested an ester of valeric or isovaleric acid. This interpretation was supported by the accurate mass values measured using GC/TOF–MS: $C_5H_{11}O_2$ for *m/z* 103.076 (calc. 103.076), $C_5H_9O$ for *m/z* 85.064 (calc. 85.065) and $C_4H_9$ for *m/z* 57.072 (calc. 57.070). Because the molecular ion was not observed, MS data were insufficient for determination of the chemical structure of both the acid and alcohol moieties.

NMR analyses of compound A (ca 100 µg in the first methanol fraction) showed seven significant signals of $^{13}C$ and $^{1}H$ (Table 1). Signals observed at $\delta_C$ 173.62 (C-1) showed a carbonyl carbon of the acid moiety and those at $\delta_H$ 2.24 (2H)/$\delta_C$ 43.19 (C-2), $\delta_H$ 2.11 (1H)/$\delta_C$ 25.71 (C-3), and $\delta_H$ 0.97 (3H×2)/$\delta_C$ 22.37 (C-4) supported the chemical structure for an ester of isovaleric acid. As for the alcohol

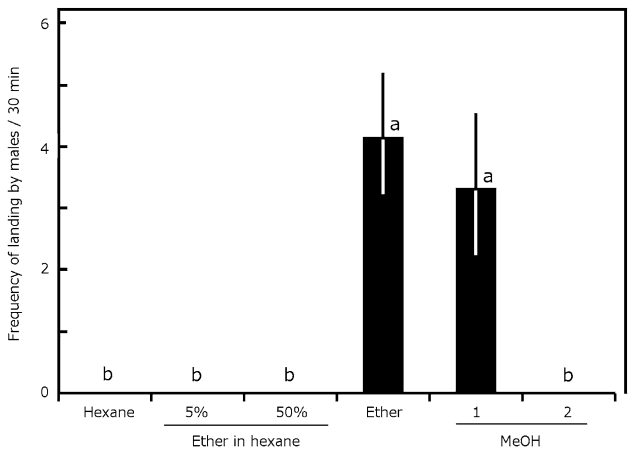



**Fig. 2** Landing by males of *Protaetia pryeri pryeri* on cotton balls treated with fractions eluted from silica gel column chromatography of solvent extracts from petals of *Luisia teres* (mean ± SE, $N = 8$). Urasoe, Okinawa, Japan, 30 June 2017. Means accompanied by the same letter are not significantly different ($p < 0.05$)

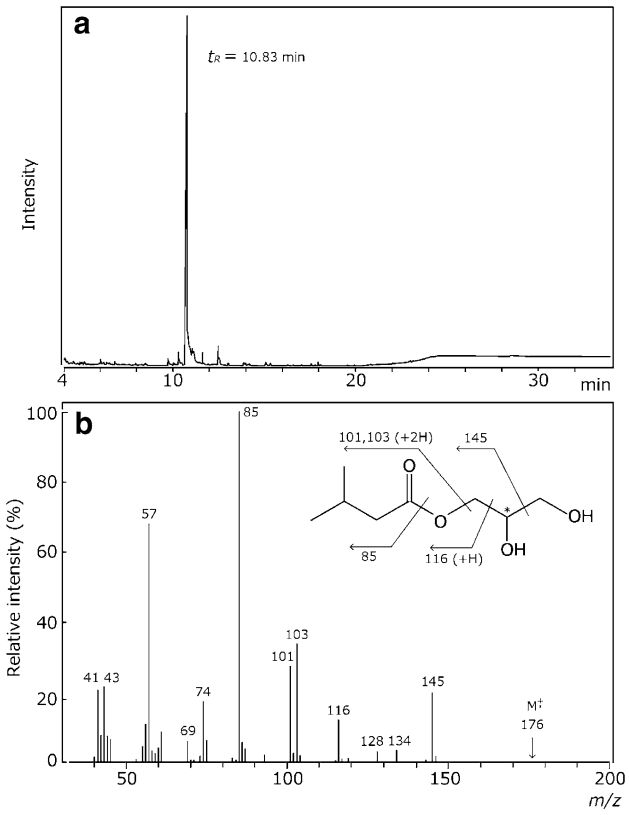
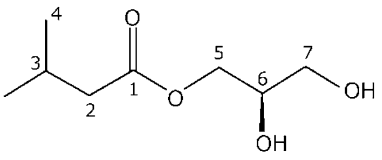



**Fig. 3** GC/MS analysis of the methanol fraction from silica gel column chromatography of solvent extracts of petals of *Luisia teres*. **a** Gas chromatogram, HP-5MS 30 m, 50 °C (1 min)–10 °C/min–280 °C (10 min). See text for details of GC condition. **b** Mass spectrum for the GC peak at $t_R = 10.83$ min [KI(HP-5MS) = 1334]. Molecular ion was not detected at *m/z* 176

**Table 1** $^{1}H$ and $^{13}C$ NMR data for compound A

| C/H no. | $\delta_H$ [ppm] (int, multiplicity, *J* in Hz) | HMBC (C) | $\delta_C$ [ppm] |
|---|---|---|---|
| 1 | | | 173.62 |
| 2 | 2.24 (2H, d, 7.2) | 1,3,4 | 43.19 |
| 3 | 2.11 (1H, sept t, 6.7, 7.2) | 1,2,4 | 25.71 |
| 4 | 0.97 (3H×2, d, 6.7) | 1,2,3 | 22.37 |
| 5 | 4.16 (1H, dd, 11.7, 6.2) | 1,6,7 | 65.10 |
| | 4.22 (1H, dd, 11.7, 4.5) | | |
| 6 | 3.94 (1H, dddd, 6.2, 5.8, 4.5, 4.0) | 5,7 | 70.29 |
| 7 | 3.61 (1H, dd, 11.5, 5.8) | 5,6 | 63.34 |
| | 3.70 (1H, dd, 11.5, 4.0) | | |




Compound A

moiety, signals observed at $\delta_H$ 4.16 and 4.22 (2H)/$\delta_C$ 65.10 (C-5), $\delta_H$ 3.94 (1H)/$\delta_C$ 70.29 (C-6), and $\delta_H$ 3.61 and 3.70 (2H)/$\delta_C$ 63.34 (C-7), as well as HMBC interpretation suggested a 2,3-dihydroxypropyl group. These interpretations were consistent with the MS data (Fig. 3) and accurate mass values of fragment ions of compound A: $C_7H_{13}O_3$ for *m/z* 145.087 (calc. 145.086), $C_6H_{12}O_2$ *m/z* 116.085 (calc. 116.082).

In subsequent comparative analyses, synthetic 2,3-DHPiV and compound A showed identical MS and NMR spectra, and identical *KI* values on HP-5 and HP-INNOWax columns (KI = 1334 and 2465, respectively). Therefore, the chemical structure of compound A was determined to be 2,3-dihydroxypropyl isovalerate (2,3-DHPiV). The total amount of 2,3-DHPiV in the ether and methanol fractions was approximately 5 µg/flower.

## Stereochemistry of compound A

Since enantiomers of 2,3-DHPiV were not separated on GC equipped with a chiral column, stereochemistry of compound A was examined by fractionation with chiral HPLC and subsequent detection with achiral GC. When synthetic racemic mixture of 2,3-DHPiV (250 ng) was injected into HPLC equipped with the chiral column, (*S*)- and (*R*)-enantiomers were eluted as two completely separated peaks at $t_R = 8.15$ min and $t_R = 9.25$ min. They were detected in fractions between 8.00 and 8.50 min and between 9.00 and 9.75 min in subsequent GC analyses, respectively (Fig. 4a). When ca. 200 ng of compound A was injected into the chiral HPLC, HPLC peaks were hidden by UV-active impurities but 2,3-DHPiV was detected in fractions from 9.00 to 9.75 min but not from 8.00 to 8.50 min by GC (Fig. 4b). Thus, the stereochemistry of compound A was determined to be (*R*), while the (*S*)-enantiomer was not detected.

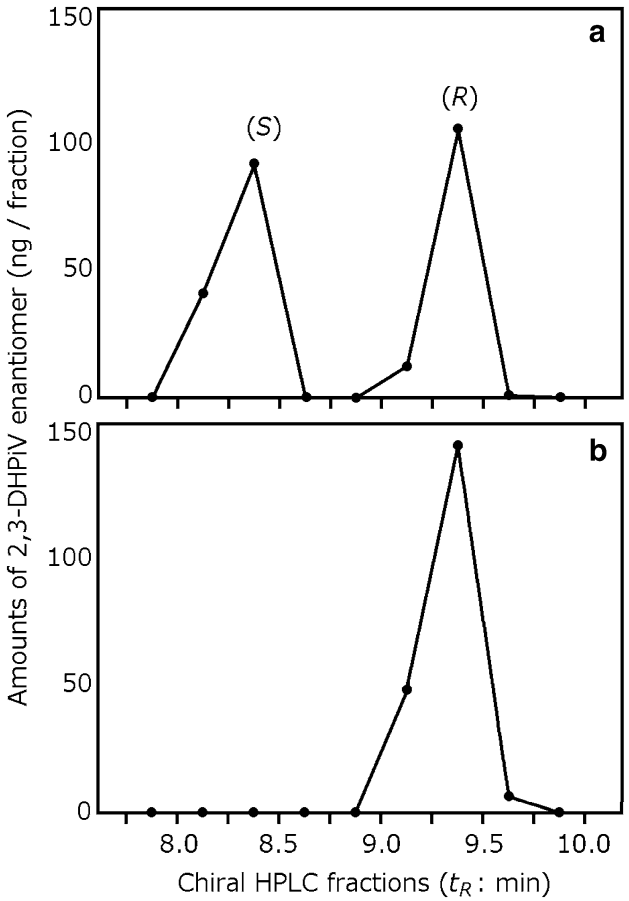



**Fig. 4** Gas chromatographic detection of enantiomers of 2,3-dihydroxypropyl isovalerate in fractions from chiral HPLC. **a** Synthetic racemic mixture (250 ng), **b** compound A (ca 200 ng) in methanol fraction of extracts from petals of *Luisia teres*. HPLC: chiral CHIRALPAK® IC column, GC: achiral HP-INNOWax column, see text for details

## Field attraction with 2,3-DHPiV enantiomer and petal extracts

When synthetic (*R*)- and (*S*)-enantiomers and racemic mixture of 2,3-DHPiV were separately impregnated onto cotton balls and assayed in the field, feral males of *P. pryeri pryeri* were observed to land on the balls impregnated with the (*R*)-enantiomer (Fig. 5). When (*R*)-enantiomer was blended with (*S*)-enantiomer, frequency of hovering by males decreased to zero as the ratio of (*S*)-enantiomer increased to 50:50 (Fig. 6a). A similar tendency was observed in landing behavior (Fig. 6b). Frequencies of hovering and landing onto cotton balls treated with synthetic 2,3-DHPiV were not significantly different from those with the extracts of petals or whole flowers, while the extracts of petals were visited by tentatively more males than the others (Fig. 7).

On the cotton balls treated with synthetic (*R*)-2,3-DHPiV, no male was observed to extrude his copulatory organ throughout the field tests.

## Detection of 2,3-DHPiV in airborne collection from female chafers

GC/MS analysis of the combined fraction obtained from volatile collection of females showed an evident peak at $t_R = 10.57$ min (KI 1334/HP-5MS). Mass spectrum and the *KI* value were identical to those of authentic 2,3-DHPiV. In a separate GC analysis on a polar column HP-INNOWax, the retention time $t_R = 19.23$ min (KI 2465) was also equal to that for the authentic 2,3-DHPiV. The amount of 2,3-DHPiV in the airborne collection was roughly determined to be 8 ng/female by comparison of the GC peak size with a known

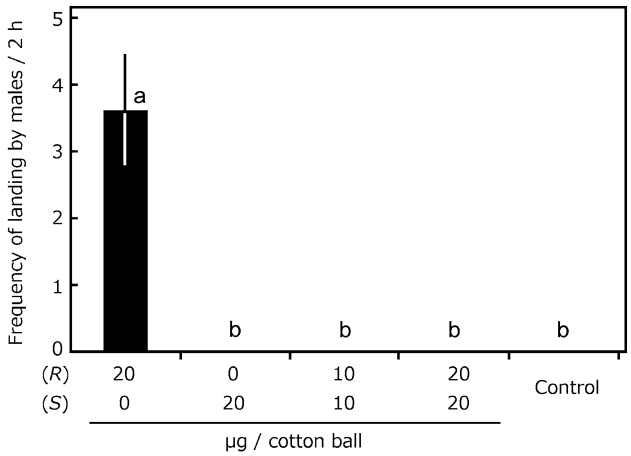



**Fig. 5** Landing by males of *Protaetia pryeri pryeri* on cotton balls treated with synthetic (*R*)- and (*S*)-enantiomers and racemic mixtures of 2,3-dihydroxypropyl isovalerate (mean ± SE, $N = 8$). Urasoe, Okinawa, Japan, 24 and 25 June 2017. Confidence interval of the mean for (*R*)-enantiomer does not contain zero ($p < 0.05$)

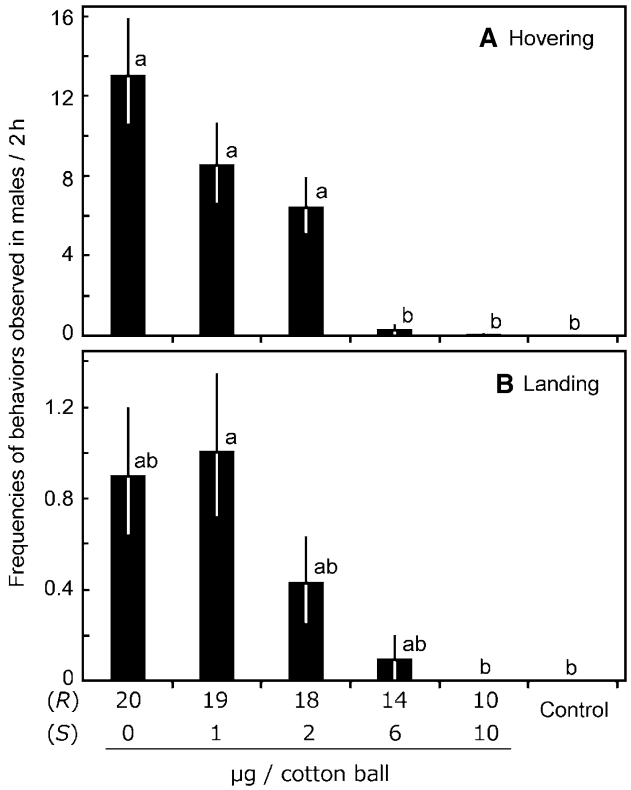



**Fig. 6** Hovering and landing by males of *Protaetia pryeri pryeri* on cotton balls treated with (*R*)- 2,3-dihydroxypropyl isovalerate and mixtures with (*S*)-enantiomer of different ratios (mean ± SE, $N = 16$). Urasoe, Okinawa, Japan, 13, 14 and 15 July 2018. Means accompanied by the same letter are not significantly different ($p < 0.05$)

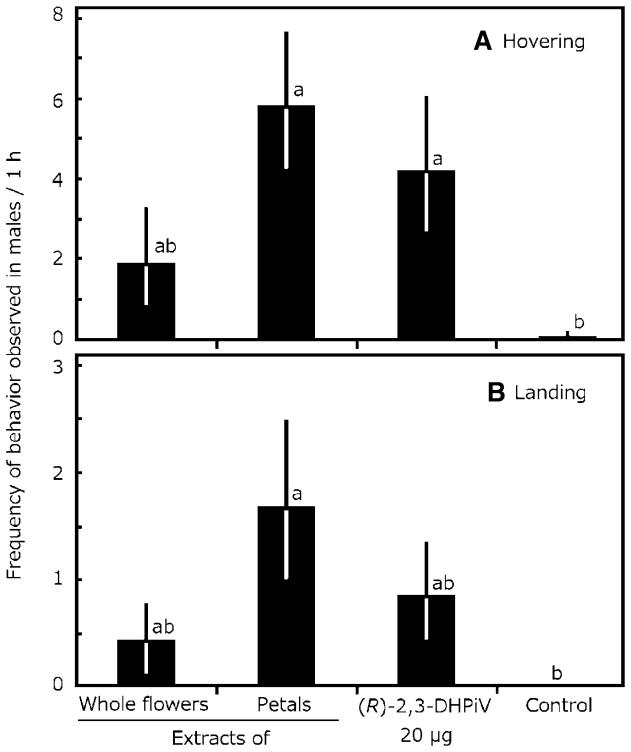



**Fig. 7** Hovering and landing by males of *Protaetia pryeri pryeri* on cotton balls treated with extracts of whole flowers and petals from each of the four flowers of *Luisia teres*, and synthetic (*R*)-2,3-dihydroxypropyl isovalerate (mean ± SE, $N = 8$). Urasoe, Okinawa, Japan, 27 June 2019. Means accompanied by the same letter are not significantly different ($p < 0.05$)

amount of authentic 2,3-DHPiV. This compound was not detected in the samples from males.

When the airborne sample was injected into HPLC with the chiral column, HPLC peaks were masked by UV-absorbing impurities. Subsequent detection using GC, however, showed no evident peak of 2,3-DHPiV probably due to loss during concentration before GC injection.

## Discussion

In a previous study (Arakaki et al. 2016), we observed that flowers of an epiphytic orchid *Luisia teres* attracted male adults of the cupreous polished chafer *Protaetia pryeri pryeri* in Okinawa, Japan. Bohman et al. (2016) described in their review that our observation is the only known Asian example showing deception of coleopteran males by orchid flower. We confirmed this observation in the present study, and identified the attractant chemical as (*R*)-2,3-dihydroxypropyl isovalerate ((*R*)-2,3-DHPiV) by assaying solvent extracts of *L. teres* flowers and synthetic compounds in the field. This compound was found in the solvent rinse of inner surface of the glass beakers, each containing a virgin female

of *P. pryeri pryeri* housed in a small wire cage, while the enantiomeric composition remains unknown. Because the females used were not allowed to touch inner surface of the glass beakers, this compound was apparently released from the insects into the air and adsorbed on the glass surface.

The synthetic (*R*)-enantiomer attracted males of *P. pryeri pryeri* in the field (Figs. 5, 6). The attractiveness of 20 µg of (*R*)-2,3-DHPiV treated on a cotton ball was comparable to that with the extracts of petals removed from four *L. teres* flowers, which contained the same amount of (*R*)-2,3DHPiV (Fig. 7). Thus, *L. teres* flower is unlikely to release another synergistic compound for attraction of this pollinator. On the other hand, whole flower extract was tentatively less attractive than petal extracts (Figs. 1, 7). This may suggest that other parts of flowers might release any inhibitory component(s). Male chafers were attracted neither to (*S*)-enantiomer nor the racemic mixtures, which suggest that the (*S*)-enantiomer suppresses the male's response to the (*R*)-enantiomer (Figs. 5, 6). (*S*)-2,3-DHPiV was not detected in the extracts of *L. teres* flowers (Fig. 4).

2,3-DHPiV was found at ca. 6 ng/female/day in airborne samples from females of *P. pryeri pryeri*, and males of this species were evidently attracted to the (*R*)-enantiomer in the field tests (Figs. 5, 6, 7). However, instrumental determination of chirality of 2,3-DHPiV released by female remains to be conducted after solving technical difficulties in chemical analysis. Nevertheless, (*R*)-2,3-DHPiV is strongly suggested to be a sex-attractant pheromone in this species.

(*R*)-2,3-DHPiV is new as a natural compound from floral scent and insect airborne pheromone to our best knowledge (Knudsen et al., 2006; Pherobase, http://www.pherobase.com/). While 2,3-DHPiV is a simple ester of rather ubiquitous compounds, glycerol and isovaleric acid, and has been synthesized by Gilchrist and Schuette (1931), information on the chemistry and biology is very scanty. Suerbaev et al. (2013) noted that 2,3-DHPiV has bactericide and antifungal activities, but information is still limited.

(*R*)-2,3-DHPiV was released not only from *L. teres* flowers but also from sexually mature female of *P. pryeri pryeri*, while chirality of the latter was not determined. These results clearly demonstrated that (*R*)-2,3-DHPiV is responsible for biological activity of floral scent of *L. teres* and very likely serves as a sex pheromone of *P. pryeri pryeri* attracting the males. From these observations, it may be concluded that *L. teres* orchids attract males of *P. pryeri pryeri* for pollination by releasing a compound that is used as the sex pheromone by the latter. The males attracted assumed the mating posture on the lip of the flower (Arakaki et al. 2016), indicating that *L. teres* is a sexually deceptive orchid. On the cotton balls treated with synthetic (*R*)-2,3-DHPiV, however, males were not observed to show pseudocopulation behavior like extrusion of copulatory organ. In addition to this fact, only 6–22% of hovering males landed on the cotton balls containing synthetic (*R*)-2,3-DHPiV (Figs. 6, 7), while ca. 50% did on those of extracts of whole or petals of flower (Fig. 7). Another additional chemical, visual and/or tactile cue(s) would be involved in release landing and pseudocopulation behavior on the flower by males.

Pedersen et al. (2013) indicated that the beetles *Lema unicolor* (Chrisomelidae) and *Clinteria ducalis* (Scarabaeidae) were attracted to unpleasant floral scent of an orchid, *Luisia curtisii*. However, they did not clearly discuss the relationship between their indication and the unpleasant smell of the flower. They provided no behavioral evidence that these beetles were attracted to the floral scent of the orchid. We also noticed an unpleasant smell for *L. teres* flowers: the smell apparently originated from the lip but not from the petals. Synthetic (*R*)-2,3-DHPiV has a faint smell for the human nose. The chemical(s) with the unpleased smell from the lip of the *L. teres* flowers has not been identified but is of interest to determine if it has any function in the deception of *P. pryeri pryeri* males.

**Acknowledgements** We thank Seiji Tanaka of National Agriculture and Food Research Organization, National Institute of Agro-biological Sciences for critical comments on the manuscript, Harukazu Fukami of KUAS for invaluable suggestion for chemical analyses and Masayuki Shimatani of Okinawa Prefectural Agricultural Research Center for assistance in chemical analysis. Thanks are also due to Sumika Tanizawa, Yuka Iketani and Aoi Kawamura, and Shuma Wake of undergraduate students at KGU for assistance in GC/MS analyses, syntheses of candidate compounds, and in the field experiments, respectively. We also thank Ikuko Maeda of the Advanced Analysis Center, NARO, for the measurement of NMR spectra.

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