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ORIGINAL PAPER

Female sex pheromone and male behavioral responses of the bombycid moth *Trilocha varians*: comparison with those of the domesticated silkmoth *Bombyx mori*

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Abstract Analysis of female sex pheromone components and subsequent field trap experiments demonstrated that the bombycid moth *Trilocha varians* uses a mixture of (*E*,*Z*)-10,12-hexadecadienal (bombykal) and (*E*,*Z*)-10,12-hexadecadienyl acetate (bombykyl acetate) as a sex pheromone. Both of these components are derivatives of (*E*,*Z*)-10,12-hexadecadienol (bombykol), the sex pheromone of the domesticated silkmoth *Bombyx mori*. This finding prompted us to compare the antennal and behavioral responses of *T. varians* and *B. mori* to bombykol, bombykal, and bombykyl acetate in detail. The antennae of *T. varians* males responded to bombykal and bombykyl acetate but not to bombykol, and males were attracted only when lures contained both bombykal and bombykyl acetate. In contrast, the antennae of *B. mori* males responded to all the three components. Behavioral analysis showed that *B. mori* males responded to neither bombykal nor bombykyl acetate. Meanwhile, the wing fluttering response of *B. mori* males to bombykol was strongly inhibited by bombykal and bombykyl acetate, thereby indicating that bombykal and bombykyl acetate act as behavioral antagonists for *B. mori* males. *T. varians* would serve as a reference species for *B. mori* in future investigations into the molecular mechanisms underlying the evolution of sex pheromone communication systems in bombycid moths.

Keywords Silkworm · Bombykol · Bombykal · Bombykyl acetate · Bombycidae · Pheromone communication system

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Introduction

Female moths produce species-specific sex pheromones to attract conspecific males. The domesticated silkworm *Bombyx mori* has long been used as a model organism for studying insect pheromone communication (Matsumoto et al. 2007; Matsumoto 2010). The first sex pheromone was isolated from *B. mori* and identified as (*E*,*Z*)-10,12-hexadecadienol (E10,Z12–16:OH; bombykol; Fig. 1; Butenandt et al. 1959). Since then, sex pheromones of more than 500 moth species have been chemically identified (see Pherobase, http://www.pherobase.com/). Studies on the control of pheromone production and machinery for pheromone biosynthesis, for example, elucidation of the pheromone biosynthesis-activating neuropeptide signaling pathway (Hull et al. 2004; Matsumoto et al. 2010) and identification of key enzymes for pheromone biosynthesis in the pheromone

gland (PG) (Moto et al. 2003, 2004), have been conducted using *B. mori*. In addition, sex pheromone receptors were identified and characterized for the first time in *B. mori* (Sakurai et al. 2004; Nakagawa et al. 2005).

B. mori females produce (*E*,*Z*)-10,12-hexadecadienal (bombykal; E10,Z12-16:Ald; Fig. 1) in addition to bombykol (Kaissling et al. 1978). Although these components are released by females and detected by males (Butenandt et al. 1959; Kaissling et al. 1978; Nakagawa et al. 2005), bombykol alone is sufficient to trigger complete mating behavior in males. Rather, it has been suggested that bombykal has an inhibitory effect on mating behavior (Kaissling et al. 1978). Regarding detection of pheromone components, *B. mori* males express two male-specific pheromone receptors (BmOR1 and BmOR3) in the olfactory sensilla, each of which responds to a specific component in a highly sensitive manner, i.e., BmOR1 responds to bombykol and BmOR3 to bombykal (Sakurai et al. 2004; Nakagawa et al. 2005).

Despite the advancement in understanding pheromone communication, important questions such as how the production of sex pheromone components has diverged during the evolution of moth species and how changes in sex pheromones have been tracked by the pheromone recognition system of male moths remain to be solved. Given the availability of complete genome data (The International Silkworm Genome Consortium 2008; Shimomura et al. 2009) and accumulated knowledge obtained through previous studies (Matsumoto et al. 2007; Matsumoto 2010), *B. mori* and its allied species can serve as a powerful model system for addressing these questions in evolutionary biology. However, although the main sex pheromone components of two wild bombycid moths, *Bombyx mandarina* (Kuwahara et al. 1983) and *Rondotia menciana* (Dai et al. 1988), have been identified, the diversity of pheromones of bombycid moths remains largely unknown. This study was part of a research project exploring the sex pheromones of several allied species of *B. mori*.

In this study, we report the sex pheromone of the bombycid moth *Trilocha varians*. This species occurs in a broad area of South Central and Southeast Asia and is a major insect pest of plants belonging to the genus *Ficus*, for example, *Ficus benjamina* (weeping fig) and *Ficus microcarpa* (Chinese banyan). First, we describe the temporal changes in occurrence of female calling behavior and identification of PG in *T. varians*. We then demonstrate that *T. varians* uses a mixture of bombykal and (*E*,*Z*)-10,12-hexadecadienyl acetate (E10,Z12-16:OAc; bombykyl acetate) as a sex pheromone. Lastly, we characterize the antennal and behavioral responses of *B. mori* to the three sex pheromone components identified so far in bombycid moths, i.e., bombykol, bombykal, and bombykyl acetate, and compare them with those of *T. varians*.

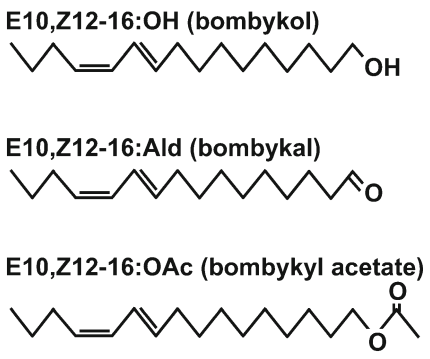

Fig. 1 Structures of chemicals used in this study. (*E*,*Z*)-10,12-hexadecadien-1-ol (E10,Z12-16:OH; bombykol). (*E*,*Z*)-10,12-hexadecadienal (E10,Z12-16:Ald; bombykal). (*E*,*Z*)-10,12-hexadecadienyl acetate (E10,Z12-16:OAc; bombykyl acetate)

Materials and methods

Insects

T. varians larvae were collected from *F. microcarpa* trees in Taipei City (25.01° N, 121.54° E), Taiwan, and Ishigaki Island (24.36° N, 124.13° E), Japan. The Ishigaki population was maintained in containment facilities at the University of Tokyo, Japan. They were reared on leaves of *F. microcarpa* or *F. superba* at 25°C under a 12-h light (L1–L12) and 12-h dark (D1–D12) photoperiod as described previously (Daimon et al. 2012). *B. mori* strain p50T, maintained in the University of Tokyo, was reared on mulberry leaves, and p50, maintained in the National Institute of Agrobiological Science, was reared on an artificial diet as described previously (Daimon et al. 2003).

Observations of moth eclosion and female calling behavior

For observation of circadian patterns of moth emergence (both sexes) and calling behavior of female moths, the pupae were individually kept in Petri dishes (90 mm diameter × 13 mm depth) under a 12-h light and 12-h dark photoperiod. A female was regarded as exhibiting calling behavior when she extruded her abdominal tip. Calling behavior of female *T. varians* moths that emerged by 1 h before lights off was observed hourly for 2 days. A dimmed red light was used for visual observation during the dark period.

Chemical analysis

A racemic mixture of Δ10,12-16:OH was purchased from Shinetsu Chemical Co. (Tokyo, Japan), and bombykol was purified from this racemate by S. Matsuyama (Tsukuba University). Authentic standards of bombykal and bombykyl acetate were synthesized from bombykol by oxidation using pyridinium chlorochromate and acetylation using

acetic anhydride, respectively. Hexadecyl acetate (16:OAc) was synthesized by acetylation of hexadecanol (Kanto Chemical). The purity of each standard was checked using a gas chromatograph (GC-17; Shimadzu; isomeric purity >99.5%). To extract the sex pheromone of *T. varians*, abdominal tips (section A + B + C; see Fig. 5) of 1-day-old female moths were excised at D2 and immersed in hexane (100 μl/tip) for 20 min at room temperature. Pheromone extracts were analyzed by a GC–mass spectrometer (QP5050 GC–MS; Shimadzu) equipped with a DB-Wax column (0.25 mm i.d.×30 m, J&W Scientific). The injector and ion source were maintained at 230°C, and the ionization voltage was 70 eV. Helium (1.0 ml/min) was used as the carrier gas. The column oven temperature was held at 120°C for the first 2 min, increased at 12°C/min to 180°C, and then at 5°C/min to 240°C.

GC–electroantennographic detection analysis

Pheromone extracts were analyzed using a GC (HP5890, Agilent Technologies) equipped with a flame ionization detector (FID) and electroantennographic detector (EAD; Taiyo Corp., Ibaraki, Japan) to determine which component(s) of the PG extract can be detected by antennae. GC conditions were the same as GC–MS conditions. The effluent from the column was evenly split between FID and EAD. An antenna was cut from a newly emerged *T. varians* male and set to the EAD device as described by Fujii et al. (2010). Antennal responses to compounds in the PG extract of *T. varians* (0.5 female equivalent) were recorded (n= 8 replications). In addition, EAD responses of *T. varians* (n=6 for both sexes) and *B. mori* (strain p50T) males and females (n=8 for males and n=3 for females) to bombykal, bombykyl acetate, and bombykol were examined by injecting a mixture of these compounds (200 ng each) into GC.

Morphology of PG in *T. varians*

Abdomens (second to sixth segments) of female moths were gently pressed with fingers to extend the abdominal tips (eighth and ninth abdominal segments). Photographs were taken with an SZX12 stereomicroscope equipped with a DP70 charge-coupled device camera (Olympus, Japan). For detailed localization of the pheromone-producing region, abdominal tips (1-day-old, D2 and L6) were cut with a razor blade to obtain tissue samples that included different sections (A + B + C, B + C, and C; see Fig. 5). A + B + C was considered as the whole abdominal tip, B + C comprised the eighth and ninth segments, and C corresponded to the ninth segment. These samples were separately immersed in hexane (300 μl), and the extracts were analyzed by GC–MS.

Behavioral analysis

Field trap experiments were performed on Ishigaki Island from April 22 to May 9, 2010. Five types of lures were prepared by impregnating rubber septa (5.3 mm o.d., Aldrich) with different mixtures of synthetic bombykal and bombykyl acetate. In a lure, hexadecyl acetate was included as a control for bombykyl acetate. A total of 500 μg of compounds was dissolved in 200 μl of hexane, applied to each rubber septum, and air-dried. No antioxidants were included in the present study. A single lure was placed at the center of each roofed sticky trap (Sankei Chemical, Japan). Traps were singly hung on *F. microcarpa* trees at a height of approximately 1.5 m, with at least 5 m of space between the traps and at least 100 m of space between replicates (n=5). The traps were left in the field throughout the test period without changing their positions. Trap data were transformed to $\log_{10}(x+1)$ and subjected to one-way analysis of variance (ANOVA) followed by Tukey's test.

To examine the behavioral responses of *B. mori* (strain p50) males to synthetic compounds, five newly emerged males were placed in a plastic container (22 cm width× 30 cm depth×6 cm height), and a piece of filter paper loaded with a defined mixture of bombykol, bombykal, and bombykyl acetate was then placed at the center of the box. Behavioral responses of the moths were observed for 5 min, and the number of moths that showed wing fluttering was counted (n=6–10 replications). Proportions of moths that showed wing fluttering were arcsine-transformed and analyzed by one-way ANOVA followed by Tukey's test.

Results

The emergence time and female calling behavior of *T. varians*

We first investigated the emergence time of *T. varians* males and females (Fig. 2). The majority of *T. varians* emerged approximately 1–2 h before the onset of the scotophase (Fig. 2b), and no apparent differences in the emergence time were observed between the sexes. We then observed the calling behavior of female moths. On the day of emergence, the calling behavior of females started 1–2 h into the scotophase and peaked between 4 and 5 h into the scotophase (Fig. 2c). Most females ceased calling by 10 h into the scotophase. On the next day, almost all female moths started calling soon after lights off.

GC–FID–EAD and GC–MS analyses of the pheromone extract

GC–FID–EAD analysis of the crude extract of abdominal tips of *T. varians* females revealed two components

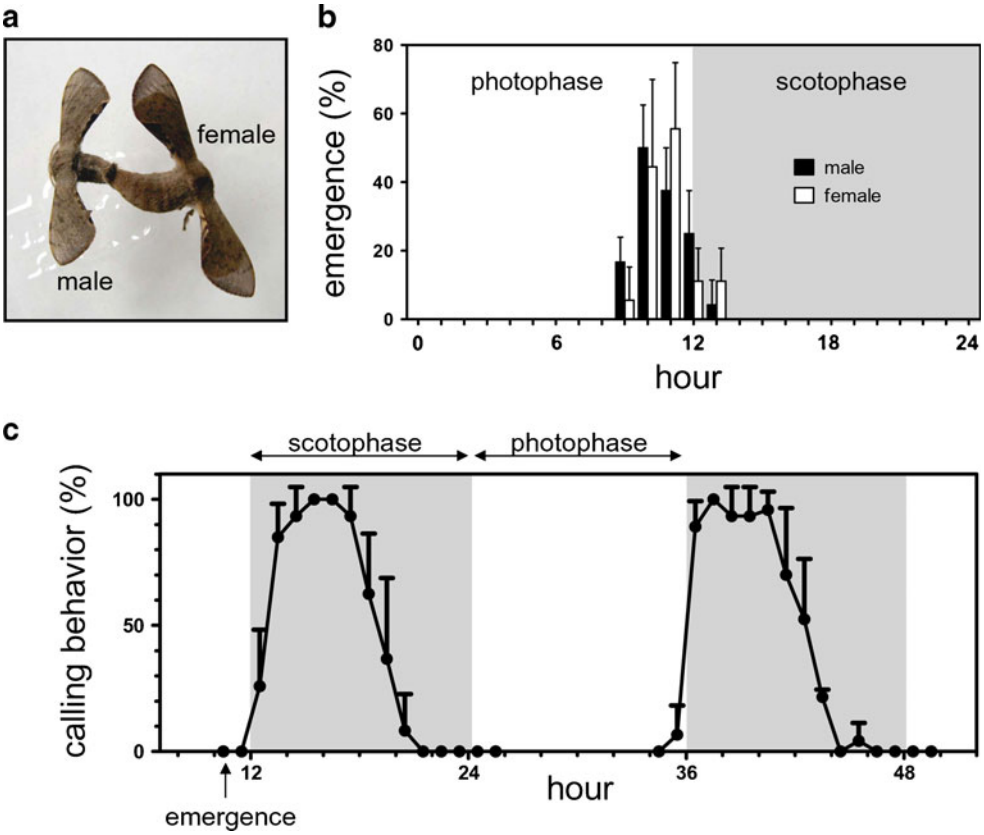

Fig. 2 Temporal changes in emergence of moths and female calling behavior of *T. varians*. **a** Copulating moths of *T. varians*. **b** Emergence pattern of *T. varians*. Fifty-four moths (n=32 for males and n=22 for females) were observed in three independent experiments (14–20 individuals/experiment). *Bars* indicate mean ± SD (n=3). *Black and white bars* represent males and females, respectively. **c** Calling frequency of *T. varians* females. Eighteen females were observed in three independent experiments (five to eight individuals/experiment). *Bars* indicate mean ± SD (n=3)

(compounds I and II; Fig. 3a) that reproducibly elicited strong responses from the antennae of *T. varians* males. We then performed GC–MS analysis of the extracts (Fig. 3b–d). On the basis of the comparison of mass fragment patterns and retention times (R_t) of compound I (base peak ion, m/z 67; molecular ion, m/z 236; diagnostic ion, m/z 207; R_t=12.93 min) and compound II (base peak ion, m/z 67; molecular ion, m/z 280; diagnostic ion, m/z 220; R_t=15.07 min) with those of authentic dienyl compounds (Ando et al. 2004), compounds I and II were identified as bombykal and bombykyl acetate, respectively (Figs. 1 and 4b). The average relative abundance of the two components was 1:2.3 (bombykal: bombykyl acetate) in three independent experiments.

Morphology of PG in *T. varians*

Female sex pheromone components of moths are synthesized in PG, which is often associated with the intersegmental membrane between the eighth and ninth abdominal segments (Fonagy et al. 2000). To determine the precise pheromone-producing region in *T. varians*, we investigated the morphology of PG in that species and compared it with the morphology of PG in *B. mori* (Fig. 4). PG in *B. mori* is a pair of sacs everted from the lateral intersegmental membrane (Fonagy et al. 2000). No such extruded organs were found in the abdominal tip of *T. varians* (Fig. 4). Eversion of PG in *B. mori* is thought to be induced by a rise in hemolymph pressure (Fonagy et al. 2000). Therefore, we pressed the abdomen of *T. varians* to raise the hemolymph pressure; however, no PG-like structures were found. One candidate for the pheromone-producing region in *T. varians* was the small membranous tissue between the eighth and ninth segments (Fig. 4d; indicated by a yellow dotted line), which is usually hidden under the sclerotized eighth segment.

Determination of the pheromone-producing region in the abdominal tip of *T. varians*

To determine the pheromone-producing region in the abdominal tip of *T. varians*, we prepared tissue samples that included different sections of the eighth and ninth abdominal segments (A + B + C, B + C, and C; Fig. 5a) and quantified the pheromone components of each sample (Fig. 5b). Section C, which is part of an ovipositor, contained little pheromone components (Fig. 5b). In contrast, section B + C contained a large amount of pheromone components; however, inclusion of section A did not increase the quantity detected (Fig. 5b; comparison of A + B + C with B + C). Taken together, this evidence indicated that the small membranous tissue between the eighth and ninth segments was likely the pheromone-producing region in *T. varians* (Figs. 4d and 5a; indicated by yellow lines).

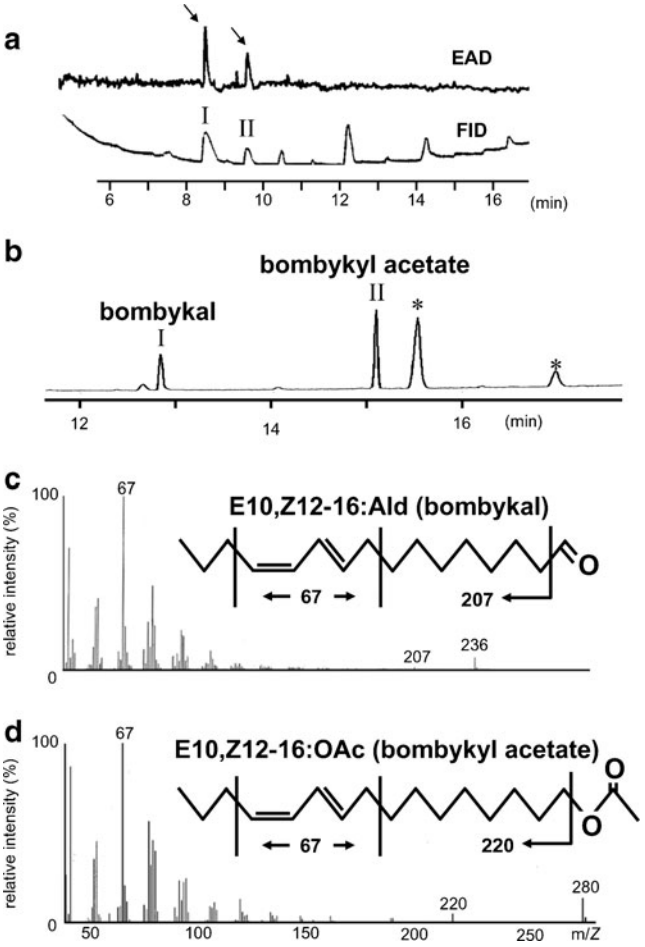

Fig. 3 GC–FID–EAD and GC–MS analyses. **a** Typical recording from GC–FID–EAD analysis of a crude pheromone gland extract of *T. varians* (0.5 female equivalent). EAD responses to two components (compounds I and II) are indicated by *arrows*. Similar results were obtained in eight independent analyses. **b** Total ion chromatogram of the PG extract of *T. varians* (0.4 female equivalent of pooled extracts from eight female moths). Compound I (R_t=12.93 min) and compound II (R_t=15.07 min) were identified as bombykal and bombykyl acetate, respectively, on the basis of comparisons of their retention times and mass fragment patterns with those of authentic standards. *Asterisks* indicate hydrocarbon peaks. Bombykol (R_t=16.23 min under the same GC–MS analysis conditions) was not detected in the PG extract of *T. varians*. **c** Mass spectrum of bombykal (identical with compound I). Base ion, 67 (100%); molecular ion, 236 ($[M]^+$, 6.8%); and diagnostic ion, 207 ($[M–29]^+$, 3.4%). **d** Mass spectrum of bombykyl acetate (identical with compound II). Base ion, 67 (100%); molecular ion, 280 ($[M]^+$, 13.5%); and diagnostic ion, 220 ($[M–60]^+$, 5.4%)

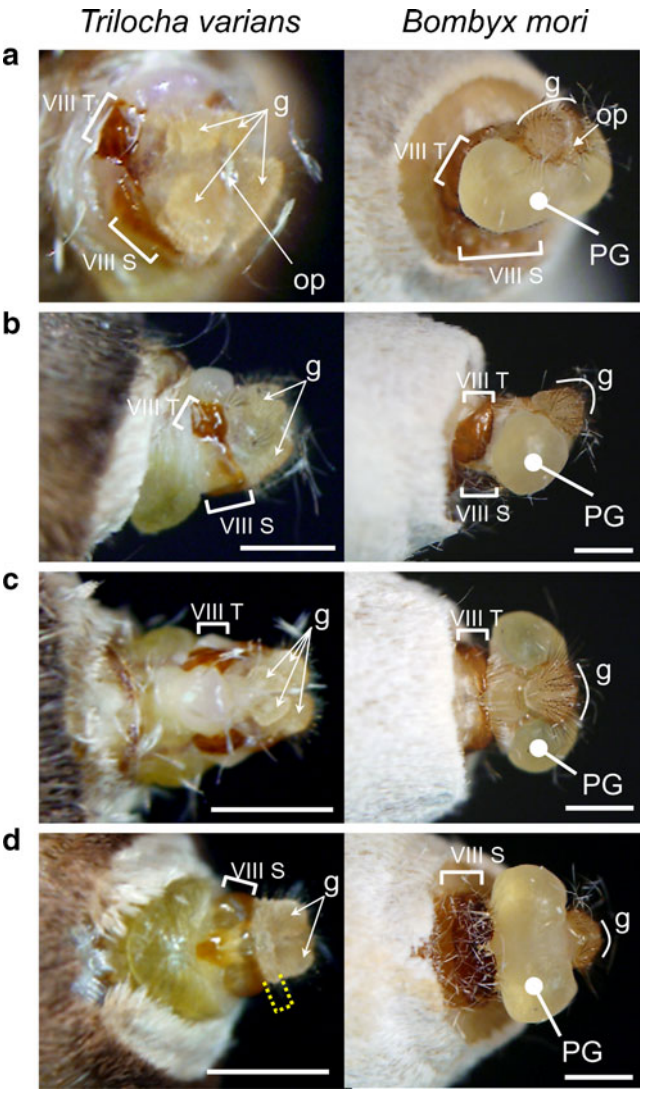

Fig. 4 Morphology of PG in *T. varians* and *B. mori* (strain p50T). **a** Extended abdominal tips of *T. varians* (*left*) and *B. mori* (*right*) females showing the eighth and ninth abdominal segments. *g* genital papilla, *op* oviporus, *VIII T* eighth abdominal tergite, *VIII S* eighth abdominal sternite, *PG* pheromone gland. **b**–**d** Lateral (**b**), dorsal (**c**), and ventral (**d**) views of the extended abdominal tip of females. A putative pheromone-producing intersegmental membrane (see Fig. 5) is indicated by the *yellow dotted line*

Changes in the pheromone titer in PG in *T. varians*

The calling behavior of *T. varians* females occurred early in the scotophase (Fig. 2c). We compared the pheromone titer of 0-day-old females during the photophase (L6) and scotophase (D2; Fig. 5c). The pheromone components of the whole abdominal tip (section A + B + C) increased approximately threefold during the scotophase, which indicated that pheromone production in PG was synchronized with calling behavior.

Antennal responses of *T. varians* and *B. mori* to synthetic pheromone components

Antennae of *T. varians* males strongly responded to bombykal and bombykyl acetate, whereas those of females did not (Fig. 6a). These results were in good agreement with those of GC–FID–EAD analyses of pheromone extracts (Fig. 3), which supported the idea that bombykal and bombykyl acetate are female sex pheromone components of *T.*

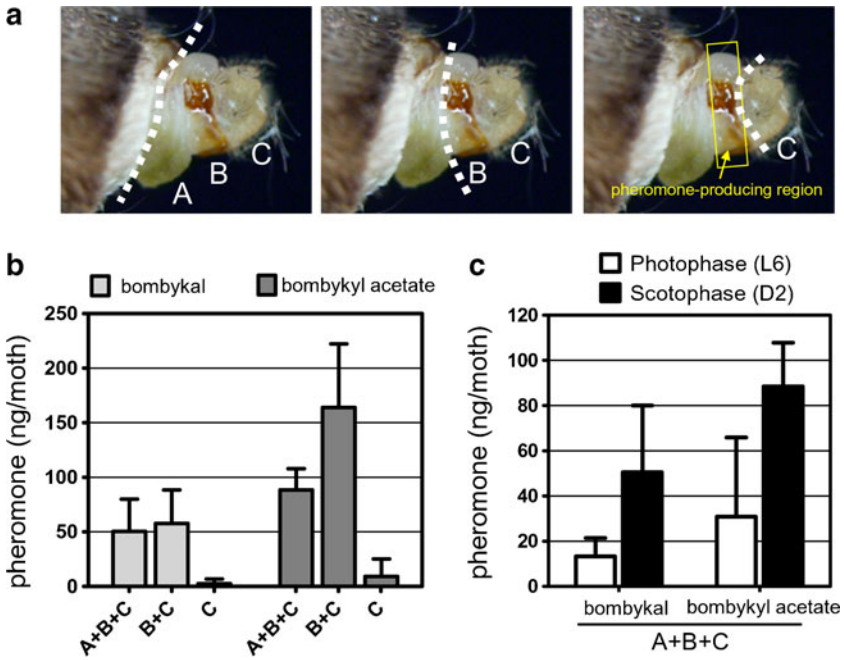

Fig. 5 Identification of the pheromone-producing region in *T. varians* females. **a** Lateral view of the extended abdominal tip of *T. varians* female. Abdominal tips were cut along *dotted lines* to locate the pheromone-producing region (A, B, or C). **b** Amount of pheromone components of regions in the abdominal tip (A + B + C, B + C, or C) sampled at 2 h into the scotophase. Samples excised from three individuals were pooled, and pheromone components were extracted in hexane and subjected to GC–MS analysis. *Bars* indicate mean ± SD (n=3 replications). **c** Changes in the pheromone titer in the abdominal tip (A + B + C) of *T. varians* females (mean ± SD, n=3). *White and black bars* represent photophase (L6) and scotophase (D2), respectively

varians. Consistent with non-production of bombykol in *T. varians*, bombykol did not elicit any response from *T. varians* antennae (Fig. 6a). This result demonstrated that the production and reception of bombykol as a sex pheromone component is not a universal trait in bombycid moths.

We then examined the antennal responses of *B. mori* to the three aforementioned compounds for comparison. As reported previously (Butenandt et al. 1959; Kaissling et al. 1978), male-specific antennal responses were elicited by bombykal and bombykol (Fig. 6b). Notably, bombykyl acetate also elicited such male-specific responses (Fig. 6b), indicating that this compound is detectable by *B. mori* males.

Field attraction of *T. varians* by synthetic lures

Based on identification of putative pheromone components of *T. varians*, we performed a field test with synthetic bombykal and bombykyl acetate. We first assumed that either of the two components would be sufficient to attract males, as in the case of *B. mori* and *R. menciana*, which are attracted to bombykol and bombykyl acetate, respectively (Butenandt et al. 1959; Kaissling et al. 1978; Dai et al. 1988; Sakurai et al. 2004). However, as shown in Fig. 7a, *T. varians* males were not attracted to bombykal or bombykyl acetate when these compounds were tested singly. Only their binary mixtures successfully attracted *T. varians* males. We tested two different blends of bombykal and bombykyl acetate (2.3:1 and 1:2.3). Although the number of males caught by the non-natural blend (2.3:1) pheromone was greater than that caught by the natural blend (1:2.3) pheromone, the difference was not statistically significant (p>0.05, Tukey's test).

Behavioral responses of *B. mori* males to bombykyl acetate

We investigated the behavioral responses of *B. mori* males to bombykyl acetate because the antennae of *B. mori* males strongly responded to this compound as well as bombykol and bombykal (Fig. 6b). As shown in Fig. 7b, neither bombykyl acetate nor bombykal evoked wing fluttering in *B. mori* males when tested singly. However, the behavioral responses of males to bombykol were significantly inhibited when bombykyl acetate was added to bombykol at a ratio of 1:1 or 1:10. The inhibitory effect of bombykyl acetate was comparable to that of bombykal (Kaissling et al. 1978). Taken together, our data clearly demonstrated that bombykyl acetate and bombykal act as behavioral antagonists for *B. mori* males.

Discussion

GC–FID–EAD and GC–MS analyses revealed that *T. varians* females produce a mixture of bombykal and bombykyl acetate as a sex pheromone (Fig. 3). To date, sex pheromones of three

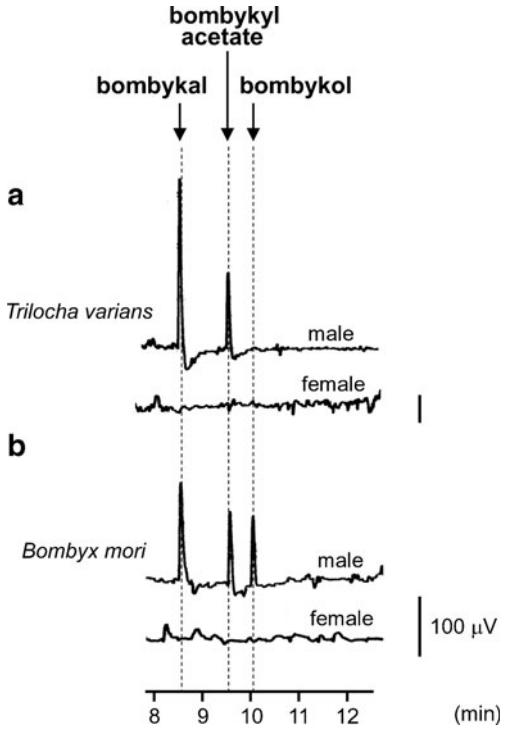

Fig. 6 EAD responses of *T. varians* and *B. mori* to pheromone components of bombycid moths. Typical responses of **a** *T. varians* and **b** *B. mori* (strain p50T) males and females (4–10 h after eclosion) to bombykal, bombykol, and bombykyl acetate (200 ng equivalent), the retention times of which are indicated by *dotted lines*. Note the male-specific responses of both species. Antennae of *B. mori* males strongly responded to bombykyl acetate, although females do not synthesize it. Experiments were performed eight times for both sexes of *T. varians*, six times for *B. mori* males, and three times for *B. mori* females. Similar results were obtained from all of these biological replications

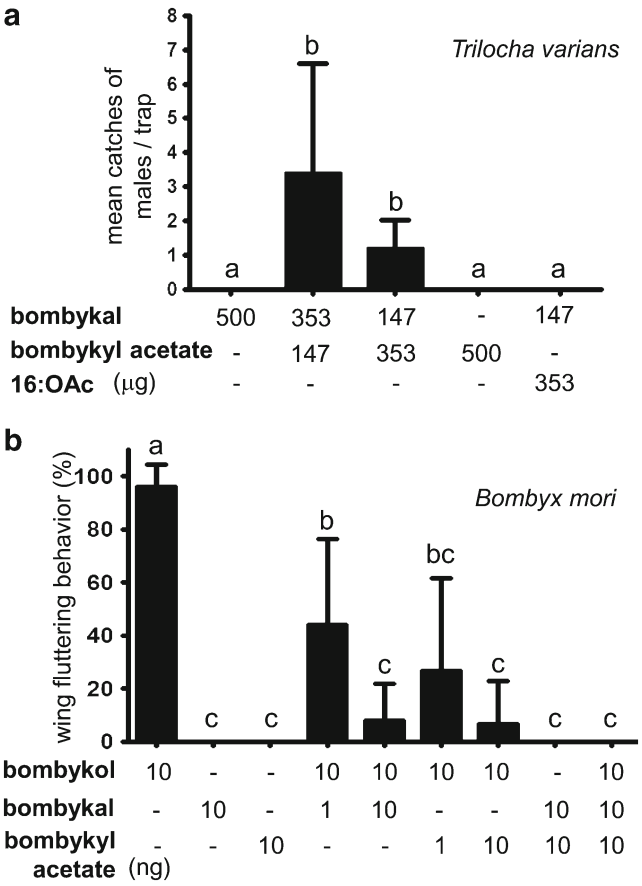

Fig. 7 Behavioral assays. **a** Number of *T. varians* males captured by lures loaded with different mixtures of bombykal, bombykyl acetate, and hexadecyl acetate (16:OAc) in a field trapping test in Ishigaki Island (mean ± SEM, five traps/lure, five replications). 16:OAc was included as a control. *Bars with different letters* are significantly different ($p<0.05$, Tukey's test). **b** Percentage of *B. mori* males (strain p50) that showed a wing fluttering response to different mixtures of bombykol, bombykal, and bombykyl acetate (mean ± SEM, five individuals/experiment, six to ten replications). *Bars with different letters* are significantly different ($p<0.05$, Tukey's test)

bombycid species have been characterized. *B. mori* females produce bombykol and bombykal (Butenandt et al. 1959; Kaissling et al. 1978), *B. mandarina* females produce bombykol (Kuwahara et al. 1983), and *R. menciana* females produce bombykyl acetate (Dai et al. 1988). In general, great diversity is created in the structure of pheromone components by varying the (a) carbon chain length, (b) number, location, and geometry of double bonds, and (c) terminal functional group (Morse and Meighen 1987). However, except for diversities in their functional groups, we did not observe structural diversities in the pheromone components of these bombycid species (Fig. 1). Previous biochemical studies have revealed the biosynthetic pathway of sex pheromone of *B. mori* in detail (Morse and Meighen 1987; Matsumoto et al. 2007; Matsumoto 2010). Fatty alcohols are important intermediates in the pheromone biosynthesis pathway in PG in *B. mori* and many other moths (Matsumoto et al. 2007; Matsumoto 2010). These alcohols are subsequently converted to corresponding aldehydes or acetates by alcohol oxidases or acetyltransferases, respectively, depending on the moth species. Our results suggested that E10,Z12-16:OH (Fig. 1; bombykol) is the common precursor of sex pheromones of *B. mori* and its allied species. Thus, the diversity of pheromone components of these bombycid species is likely to be created by turning on/off "terminal" enzymes that catalyze the oxidation or acetylation of bombykol. Aldehydes and acetate esters are widely used as pheromone components by moths (Morse and Meighen 1987). However, neither of these terminal enzymes has been cloned in any insects to date. Given the availability of the complete genome sequence of *B. mori* (The International Silkworm Genome Consortium 2008; Shimomura et al. 2009) and recent advances in next-generation sequencing technologies (Metzker 2010), comparative transcriptome analysis of PGs in *T. varians* and *B. mori* would be a promising approach to isolate these genes.

The calling behavior of *T. varians* females was frequently observed in the early- and mid-scotophase (Fig. 2c).

Furthermore, the amount of pheromone components found in the PG extract in the scotophase was greater than that in the photophase (Fig. 5c), which demonstrated circadian regulation of pheromone biosynthesis and calling behavior in *T. varians* females. Consistent with these findings, *T. varians* females were usually found to copulate with males during the early scotophase on the day of emergence and lay their eggs during the scotophase on the next day (Daimon et al. 2012).

PG in most moth species is a single layer of columnar cells located between the eighth and ninth abdominal segments. In *B. mori*, the highly enlarged PG became everted from the body during calling. In contrast, such an enlarged structure was not observed in *T. varians*, thereby showing the difference in the degree of development of PG in the subfamily Bombycinae. Our characterization of the morphology and pheromone-producing region of PG in *T. varians* (Figs. 4 and 5) will facilitate future comparative transcriptome studies on PGs in *T. varians* and *B. mori*.

In *B. mori*, information on pheromones detected by olfactory receptor neurons in the sensilla is transmitted to the large macroglomerular complex in the antennal lobe, which consists of three subdivisions, namely, the cumulus, toroid, and horseshoe (Kanzaki et al. 2003). Previous studies have shown that receptor neurons for bombykol and bombykal project to the toroid and cumulus, respectively (Kanzaki et al. 2003). These signals are then transmitted to higher olfactory centers and eventually induce defined behavioral responses (Kanzaki et al. 2003; Seki et al. 2005). Our field trap experiments using synthetic compounds demonstrated that *T. varians* males were attracted to lures only in the presence of both bombykal and bombykyl acetate (Fig. 7a). This result suggested that processing of pheromone signals in the central nervous system of *T. varians* is distinct from that of *B. mori* and *R. menciana*, in which bombykol or bombykyl acetate alone, respectively, is sufficient to trigger mating behaviors in males (Butenandt et al. 1959; Kaissling et al. 1978; Dai et al. 1988). Consequently, investigation of electrophysiological and neuroanatomical differences among these related species can lead to greater understanding of the mechanisms by which information on species-specific combinations and blend ratios of pheromone components is eventually integrated in CNS to evoke species-specific behavioral responses.

Another intriguing finding of the present study was that the antennae of *B. mori* males strongly responded to bombykyl acetate (Fig. 6b). As this response was observed only in male antennae, it is likely that the bombykyl acetate receptor (s) is selectively expressed in male antennae. To date, five ORs (BmOR1 and BmOR3–6) have been reported to be expressed at higher levels in male antennae than in female antennae (Nakagawa et al. 2005; Wanner et al. 2007). Among these, BmOR1 and BmOR3 are tuned to bombykol and bombykal, respectively (Sakurai et al. 2004; Nakagawa et al. 2005). BmOR4, BmOR5, and BmOR6 do not respond to bombykol or bombykal, and ligands for these ORs remain to be identified (Nakagawa et al. 2005). Thus, bombykyl acetate could possibly be a ligand for these male-specific orphan OR(s). However, because activation of BmOR1 or BmOR3 by bombykyl acetate has not been investigated (Nakagawa et al. 2005), there remains a possibility that one or both of them are receptors for bombykyl acetate. Therefore, future studies are needed to reexamine the specificity of BmOR1 and BmOR3–6. Notably, *BmOR1* and *BmOR3* genes are located on the Z chromosome of *B. mori* (ZW in female and ZZ in male) (Sakurai et al. 2004; Nakagawa et al. 2005; The International Silkworm Genome Consortium 2008), where genes that confer advantages to males tend to accumulate (Arunkumar et al. 2009). Similarly, among *BmOR4–6*, only *BmOR4* is located on the Z chromosome (*BmOR5* and *BmOR6* are located on chromosomes 6 and 16, respectively; The International Silkworm Genome Consortium 2008; Shimomura et al. 2009). This may suggest the possibility that BmOR4 is the receptor for bombykyl acetate.

The electrophysiological responses of the antennae of *B. mori* males to bombykyl acetate (Fig. 6b) raised a question on whether this compound has an effect on the behavior of *B. mori* males. Our behavioral assay clearly showed that bombykyl acetate as well as bombykal strongly inhibits the wing fluttering response of *B. mori* males to bombykol (Fig. 7b). However, since *B. mori* is a highly domesticated species, it is difficult to know the ecological significance of these two behavioral antagonists. We therefore plan to address this problem using *B. mandarina*, a wild counterpart of *B. mori*. Finally, it should be mentioned that although Kaissling et al. (1978) reported that *B. mori* females produce a small amount of bombykal, we found that some *B. mori* strains do not produce bombykal (Daimon et al., unpublished data). In addition, there is a large variation among silkworm strains in the amount of bombykol in PG (Shirota et al. 1998) and responsiveness of males to bombykol (Shirota et al. 1995). It is thus of interest to investigate interstrain variations in pheromone components and antennal and behavioral responses of *B. mori*.

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