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Identification of Components of the Female Sex Pheromone of the Simao Pine Caterpillar Moth, *Dendrolimus kikuchii* Matsumura

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Abstract The pine caterpillar moth, *Dendrolimus kikuchii* Matsumura (Lepidoptera: Lasiocampidae), is a pest of economic importance on pine in southwest China. Three active compounds were detected during analyses of solvent extracts and effluvia sampled by solid phase microextraction (SPME) from virgin female *D. kikuchii* using gas chromatography (GC) coupled with electroantennographic (EAG) recording with antennae from a male moth. The compounds were identified as (5*Z,*7*E*)-5,7-dodecadien-1-yl acetate (*Z*5,*E*7-12:OAc), (5*Z,*7*E*)-5,7-dodecadien-1-ol (*Z*5,*E*7-12:OH), and (5*Z*)-5-dodecenyl acetate (*Z*5-12:OAc) by comparison of their GC retention indices, mass spectra, and EAG activities with those of synthetic standards. Microchemical reactions of gland extracts provided further information confirming the identifications of the three components. Solvent extractions and SPME samples of pheromone effluvia from virgin calling females provided 100:18:0.6 and 100:7:1 ratios of *Z*5,*E*7-12:OAc:*Z*5,*E*7-12:OH:*Z*5-12:OAc, respectively. Field behavioral assays showed that *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH were essential for attraction of male *D. kikuchii* moths. However, the most attractive blend contained these three components in a 100:20:25 ratio in a gray rubber septa. Our results demonstrated that the blend of *Z*5,*E*7-12:OAc, *Z*5,*E*7-12:OH, and *Z*5-12:OAc comprise the sex pheromone of *D. kikuchii*. The optimized three-component lure blend is recommended for monitoring *D. kikuchii* infestations.

Key Words *Dendrolimus kikuchii* · (5*Z,*7*E*)-5,7-dodecadien-1-yl acetate · (5*Z,*7*E*)-5,7-dodecadien-1-ol · (5*Z*)-5-dodecenyl acetate · Field behavioral assay · Lasiocampidae · Lepidoptera · Sex pheromone · Solid phase microextraction

Introduction

Pine caterpillars (Lepidoptera: Lasiocampidae) are a serious pest of pine in China. Until now, about eighty species from seven genera have been identified (Hou, 1987). Species in the genus *Dendrolimus* Germar, including the six principal species *Dendrolimus punctatus* (Walker), *D. tabulaeformis* Tsai et Liu, *D. spectabilis* (Butler), *D. superans* Butler, *D. houi* Lajonquière, and *D. kikuchii* Matsumura, are considered to be most harmful, with populations that frequently reach outbreak status and cause significant damage to forests throughout China (Hou, 1987). The Simao pine caterpillar moth, *D. kikuchii*, is broadly distributed across southern China and causes severe damage to *Pinus yunnanensis* Franch., *P. kesiya* var. *langbianensis* (A. Chev.), *P. massoniana* Lamb., *P. armandii* Franch., *P. thunbergii* Parl., *P. taiwanensis* Hayata, *Keteleeria evelyniana* Mast., and other trees in Anhui, Jiangxi, Hunan, Hubei, Fujian,

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Zhejiang, Jiangsu, Yunnan, and portions of several other Chinese provinces (Xiao, 1991). The number of generations per year of *D. kikuchii* varies geographically. In Simao prefecture in southern Yunnan province, *D. kikuchii* has two generations per year, but in the northern part of Yunnan province only one generation occurs (Xiao, 1991). In Simao prefecture, larvae (especially the 4th through 7th instars) cause significant damage annually to large areas of pine trees during two disjunct periods (March to May, and then August to September) reducing stand volume and resin productivity. Flights of adult *D. kikuchii* usually occur in June/July and again in September/October, which overlap with the seasonal flight periods of the broadly sympatric species, *D. houi*, in Simao prefecture.

In heavily infested areas, *D. kikuchii* is controlled mainly by insecticide applications, the use of which could be enhanced and the environmental impact minimized by the timing of insecticide sprays by monitoring with pheromone traps (Zhang et al., 2003). So far, sex pheromones have been reported for the masson pine caterpillar moth, *D. punctatus* (Zhao et al., 1993), the pine moth, *D. spectabilis* (Ando et al., 1982; Kong et al., 2003), the larch caterpillar moth, *D. superans* (Klun et al., 2000; Kong et al., 2007a), the Yunnan pine caterpillar moth, *D. houi* (Kong et al., 2007b), and the European pine moth *D. pini* (Priesner et al., 1984; Kovalev et al., 1993). Sex pheromones components that have been identified from *Dendrolimus* species are all (5*Z*,7*E*)-5,7-dodecadienes with different terminal functional groups (alcohol, acetate, propionate, and aldehyde), except for the sex pheromone of *D. houi*, which was reported as a mixture of (5*E,*7*Z*)-5,7-dodecadien-1-yl acetate (*E*5,*Z*7-12:OAc), (5*E,*7*Z*)-5,7-dodecadien-1-ol (*E*5,*Z*7-12:OH), and (5*E*,7*Z*)-5,7-dodecadienal (*E*5,*Z*7-12:Ald) (Kong et al., 2007b). In preliminary GC analyses, appreciable amounts of *Z*5,*E*7-12:OAc were observed in extracts of pheromone glands of female *D. kikuchii*, along with smaller amounts of *Z*5,*E*7-12:OH (Kong et al., 2001). However, the identities of these compounds as pheromone components remained to be confirmed. In this study, we present additional analytical data on the pheromone gland components from female *D. kikuchii*, and report on their electrophysiological and behavioral activities in the laboratory and the field.

Methods and Materials

Collection and Handling of Insects Cocoons of *D. kikuchii* were collected from the host tree, *Pinus kesiya* var. *langbianensis,* near Pu'er city, Yunnan province and maintained in a rearing room at 25±1°C, 60-80% relative humidity with a 16L:8D photoperiod until adult emergence. Newly emerged males and females were housed separately under the same conditions. Virgin females were used for preparation of pheromone extracts, whereas males were used for electroantennographic studies.

Preparation of Samples Pheromone extracts were prepared as described previously (Kong et al., 2007a, b). Briefly, individual female sex pheromone glands were excised during the peak period of calling (0–2 d-old, 4–7 h into the scotophase) and extracted about 30 min in redistilled hexane (10 µl per gland). Glands were removed before sealing the extracts in glass tubes, which were stored at −20°C until bioassay and chemical analyses. Individual pheromone gland extracts were prepared (N=25) and 5 ng of (*Z*)-12-tetradecenyl acetate (*Z*12-14:OAc) were added to each as an internal standard to quantify the pheromone components.

Volatile pheromone collection was performed by solid phase microextraction (SPME) from virgin female moths (N=3) held in groups of 3-5 in 0.02 m^3 screen cages under ambient laboratory lighting and temperature conditions. After females were at rest on the walls of the cage for at least 30 min (to ensure quiescence during sample collection), we initiated collection of volatiles on a 70 µm SPME fiber (Supelco Inc., Bellefonte, PA, USA) coated with CarboWax/divinylbenzene (CW/DVB). The fiber was positioned close to the sex pheromone gland of a virgin female (2 mm) for the entire duration of the scotophase period. For analysis by GC-MS and GC-EAD, a 1 µl aliquot of a series of straight-chain aliphatic hydrocarbons (36 ng/µl of each in dichloromethane, see below for commercial source) was added directly to the sample-loaded fiber with syringe. The purpose of these standards was to assign Kováts retention indices (KI) to the components of the crude extract.

Chemical Standards Chemicals used in our analysis, e.g., all geometrical isomers of 5,7-dodecadien-1-yl acetate (*Z*5,*Z*7-, *E*5,*E*7-, *E*5,*Z*7-, and *Z*5,*E*7-12:OAc) and the corresponding alcohols (*Z*5,*Z*7-, *E*5,*E*7-, *E*5,*Z*7-, and *Z*5,*E*7-12:OH) and aldehydes (*Z*5,*Z*7-, *E*5,*E*7-, *E*5,*Z*7-, and *Z*5,*E*7-12:Ald), together with *Z*5-, and *E*5-12:OAc were purchased from Chemtech B. V. (Amsterdam, The Netherlands). *Z*12-14:OAc originated from our laboratory collection. All chemicals were purified by preparative GC using a 2 m×3.2 mm column (packed with 8% PEG 20 M on 80–100 mesh Chromosorb G-HP) in a Model 990 gas chromatograph (Perkin-Elmer Corp., Norwalk, CT, USA) as described by Kong et al. (2001, 2007b). Chemicals used as stimuli for electrophysiological and field trials were ≥99% chemically and ≥95% isomerically pure. C_{14}-C_{22} straight-chain hydrocarbons for assigning Kováts retention indices were purchased from TCI Co. (Tokyo, Japan). 4-Methyl-1,2,4-triazoline-3,5-dione (MTAD, 95%) was purchased from Aldrich Chemical (Milwaukee, WI, USA), and dimethyl disulfide (DMDS, 99%) and acetyl chloride (>99%) were purchased from Acros Organics (Geel, Belgium).

Derivatization Acetylation and methanolysis were performed to verify the conversion of alcohols (acetates) to the corresponding acetates (alcohols) (Bjostad et al., 1984). Positions of double bonds in conjugated dienes were determined by MTAD derivatization (Young et al., 1990; McElfresh and Millar, 1999), and in mono-unsaturated alkenes by the iodine-catalysed addition of dimethyl disulfide across the double bond (Buser et al., 1983). Derivatizations were performed in glass capillaries with one end sealed or in 1 ml conical vials with Teflon-lined screw caps. For acetylation and methanolysis, 14 female equivalents were evaporated to apparent dryness with a gentle nitrogen stream, then 20 µl acetyl chloride were added and allowed to react with extracted substances at room temperature for about 20 min. The product was again evaporated to dryness and re-dissolved in 6 µl of hexane. A 2 µl aliquot of the resulting solution was analyzed by GC with a flame ionization detector (FID). The remaining product of acetylation (4 µl) was evaporated again and 15 µl of KOH solution in methanol (0.5 M) were added. After 0.5 h at room temperature, 15 µl of a solution of HCl (1 M) and 30 µl hexane were added and the mixture was shaken for 2–3 min. Then 30 µl of redistilled water were added, the layers were separated and the recovered hexane portion was dried over anhydrous sodium sulfate for 24 h. After concentration with a stream of nitrogen, the solution was analyzed by GC-FID. For determination of double bond positions of conjugated dienes, MTAD derivatives of synthetic standards and compounds extracted from pheromone glands were prepared by adding 3 µl of 2 µg/µl MTAD in CH_2Cl_2 to gland extracts (7 female equivalents in 20 µl CH_2Cl_2) or to 10 ng of respective synthetic standard dissolved in 20 µl CH_2Cl_2. Mixtures were allowed to react at room temperature for 1 h. The products were concentrated and analyzed by GC-MS. For determination of double bond positions in mono-unsaturated acetates, synthetic standards and 14 female equivalent extracts in 20 µl hexane were derivatized following Buser's methods (Buser et al., 1983).

Electrophysiology The GC-EAD system consisted of a Hewlett-Packard 6890 GC equipped with flame ionization (FID) and electroantennogram (EAD) detectors. The EAD preparation consisted of a detached antenna from a 1-to 3-d-old male with the terminal segment removed, mounted directly between two EAG probes with electrically conductive gel (Spectra 360 electrode gel, Parker Laboratories Inc. Orange, NJ, USA). The signal from the EAD detector was amplified using a 10 × pre-amplifier and an AC/DC UN-6 amplifier (Syntech, The Netherlands) and fed to a personal computer equipped with an analog to digital conversion board (IDAC, Syntech). Acquisition and analysis of the EAD signals were performed by GC-EAD software (16-bit version, Syntech, The Netherlands). The GC was equipped with a split/splitless injector, a DB-WAX column (J&W Scientific, Folsom, CA, USA, 30 m× 0.25 mm i.d. × 0.25 µm) with the column effluent split by a splitter (SGE, part # OSS-2) between the FID and EAD detectors. The splitter (with a 12 ml/min N_2 make up gas to compensate for flow reduction) split the column effluent equally between the FID and EAD detectors, which were connected with the splitter by deactivated fused silica capillary columns (0.22 mm i.d.). The EAD arm was led out of the GC oven through a heated (250°C) EAD interface (Syntech) and the effluent exited into a purified and humidified airstream (~500 ml/min) directed over the antennal preparation. Synthetic compounds, solvent extracts, and SPME fibers with loaded samples were injected splitless using a temperature program starting at 80°C for 1 min, then programmed at 10°C /min to 200°C (20 min hold), N_2 was used as carrier gas, and the injector and detector temperature was set at 220°C and 250°C, respectively.

Gas Chromatography (GC) Products of acetylation and methanolysis were analyzed on an HP 5890 series II GC using a BP-20 capillary column (SGE analytical science, Brisbane, Australia, 25 m×0.22 mm i.d. × 0.25 µm) in splitless mode, temperature program: 80°C (1 min)-10°C/min-200°C (30 min). Quantitative analyses of 25 glands extracted individually were conducted on an HP6890 GC with a FID detector using a DB-WAX column (J&W Scientific, Folsom, CA, USA, 30 m×0.25 mm i.d.×0.25 µm) in splitless-mode, temperature program: 80°C (1 min)-6°C/min-200°C (10 min).

Coupled Gas Chromatography-Mass Spectrometry (GC-MS) Pheromone gland extracts and SPME samples (and all analyses for calculation of Kováts retention indices) were analyzed with a Finnigan Trace DSQ GC-MS (EI mode, 70 eV, mass range 41–460 amu) on DB-WAX and DB-5MS capillary columns (30 m×0.25 mm i.d. × 0.25 µm) with helium carrier gas (1.0 ml/min). SPME CW/DVB fibers were desorbed in the injection port for 2 min in splitless mode, program: 60°C (1 min)-6°C/min to 240°C (10 min hold), injector, ion source, and transfer line temperatures were set at 220°C, 250°C and 250°C, respectively. Full scan (TIC) and selected ion monitoring (SIM) spectra were recorded simultaneously. In SIM mode, diagnostic ions for the respective compounds were monitored. Thus, *m/z* 224 (M^+), 164 (M^+-CH_3COOH), and 136 ($C_{10}H_{16}^+$) were selected to characterize dodecadienyl acetates, *m/z* 182 (M^+), 164 (M^+-H_2O), and 121 (M^+-C_3H_9O) for dodecadienols, and *m/z* 166 (M^+-CH_3COOH) and 138 ($C_{10}H_{18}^+$) for dodecenyl acetates.

MTAD adducts were analyzed with a Finnigan Trace 2000 GC interfaced with a Voyager mass spectrometer fitted with a nonpolar HP-1 capillary column (50 m× 0.20 mm i.d.×0.33 µm; Agilent, J&W Scientific) in

splitless mode, program 80°C-15°C/min-280°C (20 min) with helium (0.6 ml/min) carrier gas in EI mode (70 eV), mass range from 41–401 amu, ion source, injector and transfer line temperatures 200°C, 280°C and 250°C, respectively. DMDS adducts were analyzed with a Finnigan Trace DSQ GC-MS (EI mode, 70 eV, mass range 41–560 amu) on a DB-5MS capillary column (30 m×0.25 mm i.d.×0.5 μm) in splitless mode. The column temperature was programmed as follows: 60°C (1 min)-6°C/min to 240°C (15 min hold). Injector, ion source, and transfer line temperatures were set at 250°C, 220°C, and 250°C, respectively. In GC-MS analysis, compounds were identified by comparison of retention indices and mass spectra with those of synthetic standards or their derivatives.

Field Behavioral Assays Lures consisted of gray rubber septa (The West Company, Phoenixville, PA, USA) loaded with hexane solutions (20 μl) of test compounds, plus 20 μl of butylated hydroxytoluene (BHT, 10 μg/μl) in dichloromethane as an antioxidant. Control septa were loaded only with 200 μg BHT in 20 μl dichloromethane. Once the treatment solutions had soaked into the septa, the lures were sealed in glass vials for transport. The lures were prepared 2 d before use and were mounted in the trap below the lid and about 2–3 cm above the sticky bottom. Experiments were set up in randomized, complete blocks with traps positioned approximately 20–30 m apart in a line and blocks separated by at least 150 m. Sticky traps, similar in shape to Pherocon 1C traps from Trécé Inc. (Salinas, CA, USA), were constructed from two pieces of cardboard (42×28 cm) with a wire in the center of the lid to suspend the septum. In traps baited with virgin females, a single virgin female moth that had emerged in the laboratory from field collected pupae was housed in a metal mesh container (8×6 cm) in place of the pheromone dispenser. Female moths were renewed every 4 d. Traps were fixed on pine tree branches ca. 1.5 m above ground, with the lures left unchanged for the entire flight period. Experiments in 2001 (eight treatments, four replicates) were designed to test male moth responses to *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH alone, and in binary combinations in different ratios. Experiments in 2007, 2009, and 2010 were designed to evaluate the effects of the trace compounds, *Z*5-12:OAc and *Z*5,*E*7-12:Ald, added to a base blend of *Z*5,*E*7-12:OAc/*Z*5,*E*7-12:OH in a 5:1 ratio. Male moths were removed and counted at 1-to 2-d intervals, depending on weather conditions during each study.

Data Handling and Statistical Analyses Kováts retention indices were calculated by using the formula described by Marques et al. (2000). For statistical analysis of field trials, the day counts for each treatment in a given block were pooled and this sum was transformed to log(x+1) to facilitate variance homogeneity prior to conducting one-way ANOVA. If significant treatment effects were detected ($P<0.05$), then the responses to the treatments were ranked by LSD *post hoc* tests ($\alpha=0.05$, SPSS13.0).

Results

GC-EAD Analyses In analyses of solvent extracts and SPME entrained volatiles from virgin female *D. kikuchii* moths by GC-EAD, up to three compounds that elicited responses from antennae of male moths were detected. The most active compound eluted at 19.33 min (Fig. 1, peak 2, Mean ± SD, 3.0±2.6 mV), and all male *D. kikuchii* antennae tested responded to this compound ($N=8$). The other two compounds that elicited antennal responses eluted earlier at 17.28 min (Fig. 1, peak 1, 0.3±0.6 mV, detected by 4 out of 8 antennae) and later at 21.09 min (Fig. 1, peak 3, 0.3±0.4 mV, detected by 3 out of 8 antennae). The GC retention times and EAD responses of the three compounds from glands agreed well with those of synthetic *Z*5-12:OAc (Mean ± SD, 2.3±1.7 mV, detected rate 100%, $N=6$), *Z*5,*E*7-12:OAc (4.0±2.9 mV, detected rate 100%, $N=6$), and *Z*5,*E*7-12:OH (0.1±0.1 mV, detected rate 33%, $N=6$), respectively, injected in 5 ng doses (2.5 ng at the antenna) during GC-EAD analyses. In addition, the antennae of male *D. kikuchii* also responded to synthetic *Z*5,*E*7-12:Ald and *E*5,*Z*7-12:Ald.

Quantitative analysis showed that single-gland extracts contained on average 1.2±0.9 ng (mean ng/female ± SD, $N=25$) of *Z*5,*E*7-12:OAc, 0.2±0.2 ng (mean ng/female ± SD, $N=25$) of *Z*5,*E*7-12:OH, and 0.01±0.05 ng (mean ng/female ± SD, $N=25$) of *Z*5-12:OAc corresponding to a ratio of 6:1:0.05.

GC-MS Analyses of Pheromone Extracts and MTAD and DMDS Adducts The first EAD-active compound EAD1 was characterized by retention indices on DB-WAX ($KI_{WAX}=1{,}909$) and DB-5 columns ($KI_{DB\text{-}5}=1{,}588$), respectively, with diagnostic ions of 166 (M^+-CH_3COOH, 14%), 138 ($C_{10}H_{18}^+$, 17%), 123 ($C_9H_{15}^+$, 11%), 110 ($C_8H_{14}^+$, 33%), and 67 ($C_5H_7^+$, 100%) (Tables 1, 2). The most abundant compound EAD2 had $KI_{WAX}=2{,}052$, and $KI_{DB\text{-}5}=1{,}636$, and was characterized by ions of *m*/*z* 224 (M^+, 4%), 164 (M^+-CH_3COOH, 23%), 136 ($C_{10}H_{16}^+$, 17%), 121 ($C_9H_{13}^+$, 28%), and 79 ($C_6H_7^+$, 100%). The third EAD-active compound EAD3 had $KI_{WAX}=2{,}149$, and $KI_{DB\text{-}5}=1{,}511$, respectively, and displayed ions at *m*/*z* 182 (M^+, 4%), 164 (M^+-H_2O, 17%), 135 (M^+-C_2H_7O, 6%), 121 (M^+-C_3H_9O, 19%), and 79 ($C_6H_7^+$, 100%). Mass spectra and retention times of the three EAD-active compounds

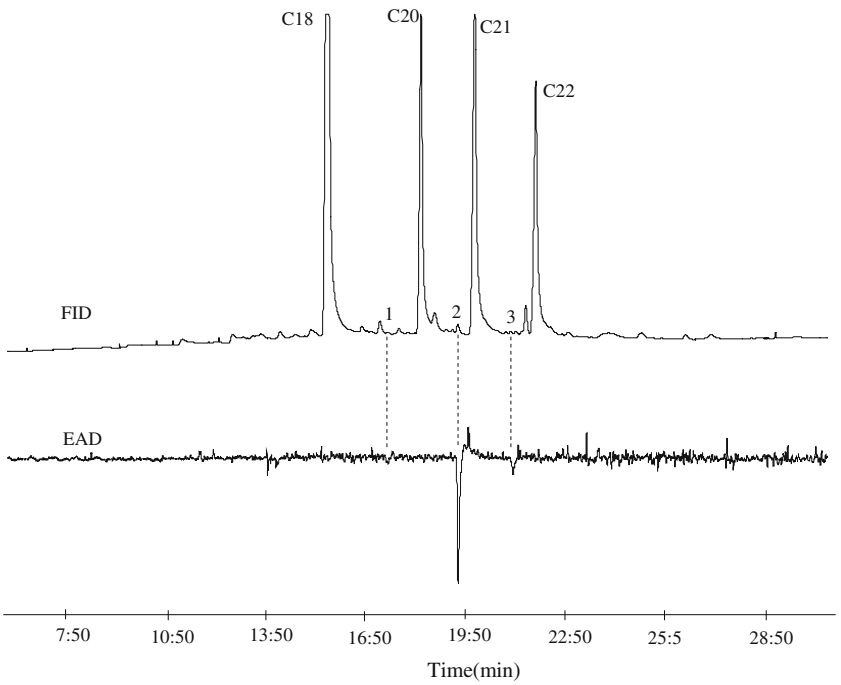

Fig. 1 Simultaneously recorded FID and EAD responses using antennae of male *Dendrolimus kikuchii* in response to sample collected by the CW/DVB fiber from one virgin female *D. kikuchii* moths. Peaks indicated by 1, 2, and 3 represent *Z*5-12:OAc, *Z*5,*E*7-12:OAc, and *Z*5,*E*7-12:OH, respectively. A 1 μl solution of hydrocarbon standards (36 ng/μl each) was added onto the fiber before injection

were identical to those of *Z*5-12:OAc (EAD1), *Z*5,*E*7-12:OAc (EAD2), and *Z*5,*E*7-12:OH (EAD3), respectively (Tables 1, 2), confirming the presence of the three compounds in the pheromone glands of *D. kikuchii*. Identities of the EAD2 and EAD3 compounds were further confirmed by microchemical analyses. Mass spectra and retention times of MTAD derivatized compounds in the gland extracts were identical to those obtained by MTAD treatment of synthetic *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH (Table 1). Conjugated double bond positions of the EAD2 and EAD3 compounds were 5,7 based on examination of diagnostic fragments from cleavage on either side of the adducts formed between the gland components and the dienophile MTAD (Table 1). In addition, acetylation and

Table 1 Electron impact mass spectral data from synthetic standards, components of pheromone gland extracts, and their MTAD and DMDS derivatives for the Simao pine caterpillar, *Dendrolimus kikuchii*

Components/Derivatives	Ions, *m/z*/abundance, % [a]				
*Z*5,*E*7-12:OAc					
Standard	224[b] /4	164[b] /20	136[b] /17	121/29	79/100
Solvent extracts	/3	/19	/16	/24	/100
SPME collections	/4	/23	/17	/28	/100
*Z*5,*E*7-12:OH					
Standard	182[b] /4	164[b] /19	135/7	121[b] /19	79/100
Solvent extracts	/4	/18	/7	/21	/100
SPME collections	/4	/17	/6	/19	/100
*Z*5-12:OAc					
Standard	166[b] /14	138[b] /18	123/9	110/35	67/100
Solvent extracts	/12	/11	/7	/22	/100
SPME collection	/14	/17	/11	/33	/100
Derivatives					
*Z*5,*E*7-12:OAc + MTAD	337/8	280/21	223/14	222/100	165/26
Solvent extracts + MTAD	/8	/21	/14	/100	/28
*Z*5,*E*7-12:OH + MTAD	295/8	238/26	222/100	181/13	165/24
Solvent extracts + MTAD	/9	/25	/100	/11	/24
*Z*5-12:OAc + DMDS	320/41	273/2	175/15	145/100	115/41
Solvent extracts + DMDS	/31	/2	/12	/100	/53

[a] Full-scan spectra obtained. MTAD, 4-methyl-1,2,4-triazoline-3,5-dione. DMDS, dimethyl disulfide

[b] Selected ion monitoring spectra obtained from solvent extracts of pheromone glands

Table 2 Compounds, Kováts retention index values, and relative quantities of pheromone candidates from calling virgin female *Dendrolimus kikuchii* analyzed by GC-MS with both DB-WAX and DB-5MS capillary columns

Compound	Kováts retention index					Relative quantity (%)		
	DB-WAX column			DB-5MS column		DB-WAX column		DB-5MS column
	standard	SPME extracts	Solvent extracts	Standard	Solvent extracts	SPME samples (Mean ± SE, *N*=3)	Solvent extracts (Mean ± SE, *N*=6)	Solvent extracts (Mean ± SE, *N*=5)
*Z*5,*E*7-12:OAc	2,052	2,052	2,052	1,636	1,636	100	100	100
*E*5,*Z*7-12:OAc	2,062	nd	nd	1,640	nd	nd	nd	nd
*Z*5,*Z*7-12:OAc	2,077	2,076	2,077	1,653	nd	0.64±0.52	0.39±0.22	nd
*E*5,*E*7-12:OAc	2,097	2,096	nd	1,666	nd	2.02±1.65	nd	nd
*Z*5,*E*7-12:OH	2,149	2,148	2,149	1,511	1,511	7.18±0.83	17.82±4.49	4.03±2.54
*E*5,*Z*7-12:OH	2,156	nd	nd	1,514	nd	nd	nd	nd
*Z*5,*E*7-12:Ald	1,875	1,874	1,874	1,436	nd	0.82±0.66	1.78±1.02	nd
*E*5,*Z*7-12:Ald	1,886	nd	nd	1,441	nd	nd	nd	nd
*Z*5-12:OAc	1,909	1,909	1,909	1,588	nd	1.01±0.83	0.57±0.33	nd
*E*5-12:OAc	1,914	nd	nd	1,591	nd	nd	nd	nd

nd not detected

base methanolysis of gland extracts resulted in disappearance and re-appearance of the alcohol peak, confirming unambiguously the OH moiety in the EAD3 compound. The mass spectral data of compounds in the DMDS derivatized gland extracts of *D. kikuchii* (Table 1) indicated the presence of the double bonds in the Δ^5 position (*m*/*z* 175 and 145) for the minor EAD1 components. The retention times matched those of the corresponding derivative from *Z*5-12:OAc, thus confirming the previous identifications. The other 5,7-isomers were excluded from consideration because their retention times were different from those of the *Z*5,*E*7-compounds. These data, along with the MTAD data, would completely prove the double bond positions and stereochemistries.

Field Behavioral Assays Preliminary field trials in 2001 had indicated that neither *Z*5,*E*7-12:OAc nor *Z*5,*E*7-12:OH was attractive alone and must be presented together (Table 3). Lures with higher doses of *Z*5,*E*7-12:OAc than *Z*5,*E*7-12:OH (9:1, 3:1 ratios) showed better attractiveness than the blends with opposite ratio (Table 3). Field experiments performed in 2007 showed that there was no significant difference in male moth captures to *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH at different ratios tested (Table 3).

Increasing the amount of *Z*5-12:OAc from 1% to 25% of the major component, *Z*5,*E*7-12:OAc, suggested an optimum level of 25% in trap captures relative to the acetate/alcohol blend (5:1 ratio) (Table 4). The same field trials conducted in 2009 and 2010 confirmed the results; the most attractive blend contained these three components in a 100:20:25 ratio released from a gray rubber septum (Table 4). The optimum synthetic pheromone blend caught more than twice as many male *D. kikuchii* moths as traps baited with a single virgin female moth (Table 4). Addition of 20 μg *Z*5,*E*7-12:Ald to either the binary blend (*Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH) or ternary blend (*Z*5,*E*7-12:OAc, *Z*5,*E*7-12:OH, and *Z*5-12:OAc) resulted in significant reductions in trap catches relative to the acetate/alcohol blend or the optimum 3-component blend (Table 4).

Table 3 Catches of male *Dendrolimus kikuchii* in traps baited with various combinations of *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH, Yunnan province, China

Composition of baits (μg)		Mean catch ± SE / trap [a] *N*=4
*Z*5,*E*7-12:OAc	*Z*5,*E*7-12:OH	
Experiment (2001)		
1000	0	0
0	1000	0
900	100	2.75±0.85 ab
750	250	7.00±2.61 a
500	500	0.75±0.48 bc
250	750	0.25±0.25 c
100	900	0
control		0
Experiment (2007)		
450	150	8.00±1.41 a
500	100	7.00±1.00 a
525	75	5.00±0.58 a
control		1.50±0.96 b

[a] Means followed by the same letter are not significantly different at the 5% confidence level by LSD tests ($F_{(3,12)}$=8.10, P<0.003 for Experiment 2001 on June 19-July 2 in Zhenyuan county; $F_{(3,12)}$=7.35, P<0.005 for Experiment 2007 on June 16–30 in Pu'er city)

Table 4 Catches of male *Dendrolimus kikuchii* in traps baited with various blends of potential pheromone components in Pu'er city, Yunnan province, China

Composition of baits (μg)				Mean catch ± SE/trap[a]		
*Z*5,*E*7-12:OAc	*Z*5,*E*7-12:OH	*Z*5-12:OAc	*Z*5,*E*7-12:Ald	Experiment (2007) *N*=4	Experiment (2009) *N*=5	Experiment (2010) *N*=4
500	100	0	0	7.00±1.78 ab	3.80±0.73 bc	4.50±1.32 bc
500	100	5	0	1.75±0.48 cd	2.60±0.68 c	1.50±0.61 c
500	100	25	0	5.00±2.42 bc	5.20±1.16 b	3.00±0.58 bc
500	100	125	0	11.00±1.29 a	9.80±1.66 a	16.50±3.77 a
500	100	250	0	–[b]	–	9.00±3.24 ab
500	100	125	20	–	0	0
500	100	0	20	1.00±0.34 d	0.80±0.37 d	–
Live female				–	4.20±0.86 bc	6.00±2.27 ab
Control				1.50±0.65 d	0	0

[a] Means followed by the same letter in each column are not significantly different at the 5% confidence level by LSD tests (Experiment 2007, June 12-July 8, $F_{(5,18)}=10.52$, $P<0.001$; Experiment 2009, June 16–30, $F_{(5,24)}=10.83$, $P<0.001$; Experiment 2010, June 11–23, $F_{(5,18)}=4.05$, $P<0.01$)

[b] "–" indicated the treatments were not conducted

Discussion

Two suspected pheromone components, *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH, have been known from *D. kikuchii* moths for quite some time but their functions were unclear (Kong et al., 2001). The present study describes efforts to clarify the female-produced sex pheromone of *D. kikuchii*, which apparently consists of at least three compounds, i.e., the primary component *Z*5,*E*7-12:OAc and the less abundant *Z*5,*E*7-12:OH and *Z*5-12:OAc. These three compounds were detected in both pheromone gland extracts and SPME entrained volatiles of calling females by GC-MS and GC-EAD methods. The antennae of male *D. kikuchii* were most sensitive to *Z*5,*E*7-12:OAc followed by *Z*5-12:OAc and *Z*5,*E*7-12:OH in GC-EAD analyses, although *Z*5-12:OAc is 20-fold less abundant than *Z*5,*E*7-12:OH in gland extracts. Following the common pattern of pheromone chemistry in the *Dendrolimus* genus, female moths of *D. kikuchii* employ the *Z*5,*E*7-configuration of the acetate and alcohol pheromone components and both components are essential for attraction. In this study, field trapping data also have provided some evidence for the behavioral role of the minor EAD-active component, *Z*5-12:OAc, confirming its status as a pheromone component of *D. kikuchii*. In addition, *Z*5-12:OAc also has been found in *D. punctatus*, although it is not part of the attractive pheromone blend of that species (Zhao et al., 1993).

SPME samples obtained by CW/DVB coated fiber (70 μm) and conventional samples obtained by solvent extraction were similar, and provided enough material for GC-MS analyses of the most abundant *Z*5,*E*7-12:OAc. Clear mass spectra were not obtained from solvent extracts of *Z*5,*E*7-12:OH and *Z*5-12:OAc because the compounds were present in much smaller quantities than in the SPME samples. SPME sampling was more effective and provided enough material for unambiguous full scan GC-MS identification. The assignments of the stereoisomers of the EAD-active peaks were completed by comparison of their retention indices with those of the synthetic acetate and alcohol on DB-WAX and DB-5MS columns. The retention times on two columns of different polarity provide strong evidence of the identity of the three compounds. In addition, the GC-MS data of MTAD and DMDS derivatization with gland extracts further confirmed the presence of *Z*5,*E*7-12:OAc, *Z*5,*E*7-12:OH, and *Z*5-12:OAc in the pheromone glands. The fact that the retention times of the other 5,7 isomers were different also confirms the stereochemistry as *Z*5,*E*7.

Female-emitted sex pheromones typically have two or more components, and component ratio often is important in evoking optimal responses from males (Linn and Roelofs, 1989). Although the response of *D. kikuchii* males broadly overlaps the component ratios found in the gland extracts of virgin females with different methods, in the species studied here, the pheromone component ratios are important as in the closely related *D. punctatus* and *D. spectabilis*, which are sympatric in northern China. *Dendrolimus kikuchii* and *D. spectabilis* are entirely allopatric and so can use *Z*5,*E*7-12:OAc and *Z*5,*E*7-12:OH as major pheromone components, *D. kikuchii* in a 100:7 (SPME) ratio and *D. spectabilis* in about the inverse ratio (Kong et al., 2003). Over much of southern China, *D. kikuchii* is broadly sympatric with *D. punctatus*, which employs a 100:75 blend of *Z*5,*E*7-12:OH and *Z*5,*E*7-12:OAc as major pheromone components (Zhao et al., 1993).

The difference in ratios between major pheromone components could serve as a premating reproductive isolation mechanism between the sympatric *D. kikuchii* and *D. punctatus*. Furthermore, the minor components, *Z*5-12:OAc and (5*Z*,7*E*)-5,7-dodecadien-1-yl propionate, function to enhance the specificity of the signals, respectively. In addition, *Z*5,*E*7-12:OAc is known to serve as a strong inhibitor of European pine caterpillar moth *D. pini* whose major pheromone component is *Z*5,*E*7-12:Ald (Priesner et al., 1984). In the current research, *Z*5,*E*7-12:Ald at relatively high dose showed strong antagonistic effects on attraction of male *D. kikuchii*, although trace quantities of it have been found in the gland extracts. From the point of electrophysiological evidence, *Z*5,*E*7-12:Ald and *E*5,*Z*7-12:Ald might be involved in the inhibition of sympatric species *D. kikuchii* and *D. houi*.

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References

ANDO, T., VU, M. H., YOSHIDA, S., TAKAHASHI, N., TATSUKI, S., KATAGIRI, K., YAMANE, A., IKEDA, T., and YAMAZAKI S. 1982. (5*Z*,7*E*)-5,7-Dodecadien-1-ol: Female sex pheromone of the pine moth *Dendrolimus spectabilis* Butler. *Agric. Biol. Chem*. 46:709–715.

BJOSTAD, L. B., LINN, C. E., DU, J. W., and ROELOFS, W. L. 1984. Identification of new sex pheromone components in *Trichoplusia ni*, predicted from biosynthetic precursors. *J. Chem. Ecol*. 10:1309–1323.

BUSER, H. R., ARN, H., GUERIN, P., and RAUSCHER, S. 1983. Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Anal. Chem*. 55(6):818–822.

HOU, T. Q. 1987. Introduction to pine caterpillars, pp. 1–26, in T. Q. Hou (ed.). The Pine Caterpillars in China. Science Press, Beijing, China (in Chinese).

KLUN, J. A., BARANCHIKOV, Y. N., MASTRO, V. C., HIJJI, Y., NICHOLSON, J., RAGENOVICH, I., and VSHIVKOVA, T. A. 2000. A sex attractant for the Siberian moth *Dendrolimus superans sibiricus* (Lepidoptera: Lasiocampidae). *J. Entomol. Sci*. 35:158–166.

KONG, X. B., ZHAO, C. H., and GAO, W. 2001. Identification of sex pheromones of four economically important species in genus *Dendrolimus*. *Chinese Sci. Bull*. 46:2077–2081.

KONG, X. B., ZHAO, C. H., SUN, Y. P. FENG, S. Q., and WU, H. S. 2003. Identification of minor components of the pine caterpillar moth, *Dendrolimus spectabilis* sex pheromone: components, electrophysiological activity, and field effects. *Acta Entomol. Sin*. 46:131–137 (in Chinese).

KONG, X. B., ZHAO, C. H., and WANG, R. 2007a. Sex pheromone of the larch caterpillar moth, *Dendrolimus superans*, from north-eastern China. *Entomol. Exp. et Appl*. 124:37–44.

KONG, X. B., ZHANG, Z., ZHAO, C. H., and WANG, H. B. 2007b. Female sex pheromone of the Yunnan pine caterpillar moth, *Dendrolimus houi*: First (*E*,*Z*)-isomers in pheromone components of *Dendrolimus* spp. *J. Chem. Ecol*. 33:1316–1327.

KOVALEV, B. G., BOLGAR, T. S., ZUBOV, P. A., ZHARKOV, D. G., GOLOSOVA, M., NESTEROV, E. A., and TVARADZE, M. S. 1993. Identification of additional components of the sex pheromone of *Dendrolimus pini*. *Chem. Nat. Compd*. (Historical Archive) 29:135–136.

LINN, C. E., and ROELOFS, W. L. 1989. Response specificity of male moths to multicomponent pheromones. *Chem. Senses*. 14:421–437.

MARQUES, F. d. A., MCELFRESH, J. S., and MILLAR, J. G. 2000. Kováts retention indexes of monounsaturated C12, C14, and C16 alcohols, acetates and aldehydes commonly found in Lepidopteran pheromone blends. *J. Braz. Chem. Soc*. 11:592–599.

MCELFRESH, J. S., and MILLAR, J. G. 1999. Sex pheromone of the common sheep moth, *Hemileuca eglanterina*, from the San Gabriel Mountains of California. *J. Chem. Ecol*. 25:687–709.

PRIESNER, E., BOGENSCHÜTZ, H., ALBERT, R., REED, D. W., and CHISHOLM, M. D. 1984. Identification and field evaluation of a sex pheromone of the European pine moth. *Z. Naturforsch*. 39c:1192–1195.

XIAO, G. Y. 1991. Forest Insects of China, pp. 935–969, 2nd eds., China Forestry Publishing House, Beijing, China (in Chinese).

YOUNG, D. C., VOUROS, P., and HOLICK, M. F. 1990. Gas chromatography-mass spectrometry of conjugated dienes by derivatization with 4-methyl-1,2,4-triazoline-3,5-dione. *J. Chromatogr*. 522:295–302.

ZHANG, A. B., WANG, Z. J., TAN, S. J., and LI, D. M. 2003. Monitoring the masson pine moth, *Dendrolimus punctatus* (Walker) (Lepidoptera: Lasiocampidae) with synthetic sex pheromone-baited traps in Qianshan county, China. *Appl. Entomol. Zool*. 38:177–186.

ZHAO, C. H., LI, Q., GUO, X. Y., and WANG, X. Y. 1993. New components of sex pheromone in the pine caterpillar moth, *Dendrolimus punctatus*: identification of chemical structures and field tests. *Acta Entomol. Sin*. 36:247–250 (in Chinese).