

# 養菌性キクイムシ、カシノナガキクイムシ(鞘翅目: ナガキクイムシ科)の集合フェロモン、(1S,4R)-p-メント-2-エン-1-オール

誌名	森林総合研究所研究報告
ISSN	09164405
著者名	所,雅彦 小林,正秀 斉藤,正一 衣浦,晴生 中島,忠一 正田・加賀谷,悦子 柏木,丈拡 手林,慎一 金,哲史 森,謙治
発行元	森林総合研究所
巻/号	6巻1号
掲載ページ	p. 49-57
発行年月	2007年3月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター  
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council  
Secretariat



## 論文 (Original article)

# Novel aggregation pheromone, (1*S*,4*R*)-*p*-menth-2-en-1-ol, of the ambrosia beetle, *Platypus quercivorus* (Coleoptera: Platypodidae)

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### Abstract

We identified an aggregation pheromone of *Platypus quercivorus* using volatiles from the boring frass of an unmated male. Gas chromatography – electroantennographic detector (GC–EAD) showed an EAD active component from the volatiles. GC – mass spectrometry (MS) data and comparison of the retention times of stereospecific synthetic compounds of the optical isomers on chiral GC column showed that the chemical structure of the EAD active component was (1*S*,4*R*)-*p*-menth-2-en-1-ol (quercivorol). A field trapping tests using quercivorol and its optical isomer demonstrated that the quercivorol is main component of aggregation pheromone of *P. quercivorus*.

**Key words :** *Platypus quercivorus*, aggregation pheromone, (1*S*,4*R*)-*p*-menth-2-en-1-ol, quercivorol, ambrosia beetle

### Introduction

*Platypus quercivorus* (Murayama)(Coleoptera: Platypodidae) is an ambrosia beetle that bore into the trunks of oaks and other broad-leaved trees in Japan, Taiwan, India, Indonesia and New Guinea (Wood & Bright, 1992). In platypodid ambrosia beetles, male-initiated monogamy is usual (Kirkendall, 1983). Mass mortality of oaks, especially *Quercus crispula* Blume, resulting when large infestations of these beetles spread Japanese oak wilt, has occurred continuously in Japan since 1934 (Hijii *et al.*, 1991; Ito & Yamada, 1998). Ito *et al.*, (1998) reported that ambrosia fungi caused the mass mortality of Japanese oak, and that *P. quercivorus* is considered to be a critical vector of the fungi. Later, a phytopathogenic fungus was isolated from the gallery of the trunk and mycangia of the beetle, and named as *Raffaelea quercivora* (Kubono & Ito, 2002).

In recent studies, concentrative boring was observed on a particular oak tree soon after the initial attack of the beetle (Ueda & Kobayashi, 2001, Kobayashi & Ueda, 2003), suggesting the presence of semiochemicals attracting a large number of individuals. Field experiments have indicated that chemical communication was responsible for mass attacks on oaks by the beetles at long range (Ueda & Kobayashi, 2005), whereas sound communication is a cue for their mating behavior at close range

as well (Ohya & Kinuura, 2001, Kobayashi & Ueda, 2002).

In some platypodid species that attack their hosts in large numbers, the males release aggregation pheromone, which attracts conspecific males and females (Madrid *et al.*, 1972; Milligan, 1982; Milligan & Ytsma, 1988; Milligan *et al.*, 1988). Sulcatol (6-methyl-5-hepten-2-ol), which is the aggregation pheromone of some scolytid beetles including *Gnathotricus sulcatus* (LeConte) (Byrne *et al.*, 1974), *G. sulcatus materiarius* Fitch (Fletchmann & Berisford, 2003) and *G. sulcatus retusus* (LeConte) (Borden *et al.*, 1979), was determined to be the main component of aggregation pheromones of platypodid beetles as *P. flavicornis* (F.) (Renwick *et al.*, 1977) and *P. wilsoni* (Shore & McLean, 1983). Although Ueda and Kobayashi (2001) suggested the presence of aggregation pheromone in *P. quercivorus*, it has not been chemically identified and biologically assayed. In this paper, we identify the aggregation pheromone of *P. quercivorus*.

### Materials and Methods

#### Test Insects

Logs of *Q. crispula* Blume infested by *P. quercivorus* were collected from oak forests in Aizu-wakamatsu, Fukushima prefecture and Keihoku, Kyoto city, Kyoto prefecture, Japan in

原稿受付：平成 18 年 12 月 11 日 Received Dec. 11, 2006 原稿受理：平成 19 年 1 月 9 日 Accepted Jan. 9, 2007

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October 2003 and 2004, and kept in a temperature-controlled room (16°C). Emerging beetles were collected daily, sexed, and kept until use of experiments.

### Chemicals

Synthetic racemic *cis-p*-menth-2-en-1-ol for 2004 and 2005 field traps test was purchased from Sankei Chemical Co Ltd. (Tokyo, Japan) as a mixture of *cis-p*-menth-2-en-1-ol (> 76.2%) and *trans*-isomer (> 17.9%) (racemic quercivorol **I**). In this paper, the *cis*-isomer is defined as the stereoisomer with both the methyl and the isopropyl groups in the same direction. Racemic *cis-p*-menth-2-en-1-ol, synthesized from ( $\pm$ )-cryptone, chemical purity was 79.8%, contains 4.1% of *trans-p*-menth-2-en-1-ol for 2006 field trap test (racemic quercivorol **II**; Mori, 2006). (1*S*,4*R*)-*p*-menth-2-en-1-ol (quercivorol: chemical purity 92.0%, optical purity 96.0%) was synthesized from (*S*)-perillyl alcohol (Mori, 2006). (1*R*,4*S*)-*p*-menth-2-en-1-ol (chemical purity 95.4%, optical purity 96.1%) was synthesized from (*R*)-limonene (Kashiwagi *et al.*, 2006). Ethyl alcohol was purchased from Nihon Alcohol Hanbai Corp. Acetone (99.8%) and *n*-hexane (96.0%) and all other reagents were purchased from Wako Chemical Ltd. and Nacalai Tesque Ltd. in Japan.

### Collection of volatiles

Logs of *Q. serrata* Thunb. ex Murray uninfested by *P. quercivorus* (20–40 cm long, 10–20 cm i.d.) were soaked in water for over ten days to improve the breeding condition of the beetles (Kitajima & Goto, 2004).

Holes (2 mm i.d. and 1–1.5 cm depth) were bored by drill

into the logs at 2–5 cm intervals. Unmated male beetles were introduced as pioneers into the holes through a plastic pipette chip (1 ml volume), with tips cut to allow the beetle to pass into the logs. Each hole occupied by one male beetle. Within several hours, the male beetles started to push out the frass to the surrounding tunnel entrances. And after a while, boring the males deposited droplets from their anuses onto the boring frass circles. Similar behaviors were reported in males of *P. apicalis* and *P. gracilis* (Milligan & Ytsma, 1988). After four days, the masses of boring frass from single tunnel entrance were collected at 10:00 a.m. and put into glass screw vials (1.5–5 ml). Volatiles from the head space of the vials were collected using a solid phase microextraction (SPME) holder with a fiber of 100  $\mu$ m polydimethylsiloxane (Supelco, Bellefonte, PA, USA) for about six hours. To extract more volatile components, boring frass from 30 tunnel entrances of unmated male beetles were soaked in 3 ml acetone for over one day. An unmated male or female beetle newly emerged was also soaked in 150  $\mu$ l of hexane for over one day for extraction.

### Analysis of volatile components

#### Gas chromatographic–electroantennographic detection (GC–EAD)

Volatiles collected from the acetone boring frass extracts or SPME fiber were analyzed by GC–EAD system described by Chen *et al.*, 2006. A Hewlett-Packard 5890 series II GC was equipped with a nonpolar column HP1-MS (30 m  $\times$  0.25 mm i.d.  $\times$  0.25  $\mu$ m film thickness, J&W Scientific, Folsom, CA, USA), a polar column DB-23 (30 m  $\times$  0.25 mm i.d.  $\times$  0.25  $\mu$ m film thickness, J&W Scientific), or a chiral separation

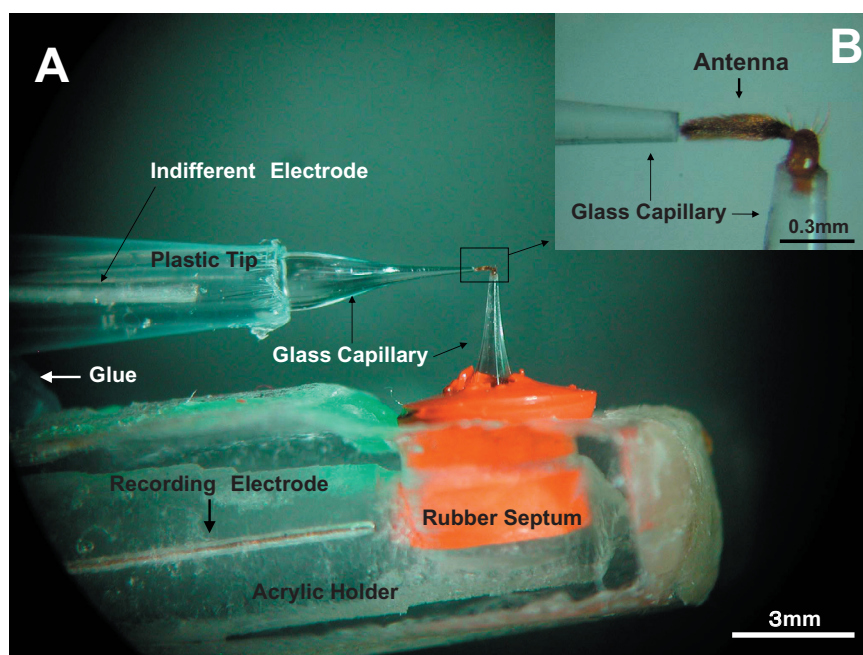


Fig. 1. A: Acrylic antenna holder with electrodes for EAD preparation  
B: Close-up view of antenna. See text for details.

column CP-Chirasil-Dex CB (25 m × 0.25 mm i.d. × 0.25 µm film thickness, Chrompack, London, UK). The temperatures of the injection port and flame ionization detector (FID) port were 220°C and 250°C, respectively. Oven temperatures were programmed at 50°C for 1 min, then 10°C/min to 250°C for the non-polar column, 60°C for 1 min, then 15°C / min to 200°C for the polar column, 60°C for 1 min, then 10°C / min to 200°C for the chiral column. For SPME sample injection, a 0.75 mm diameter glass inlet liner (Supelco) was used, and for acetone extracts sample injection, a 3 mm diameter glass inlet liner was used for all column types.

An antenna including basic segment was gently pulled out from a live beetle with tweezers under a stereoscopic microscope, and was placed between recording and indifferent electrodes in an acrylic holder (Fig. 1). The acrylic holder that we used was modified from the method of Nojima *et al.*, (2003) for tiny antennae from small beetles, using a 1.5 mm diameter glass capillaries (tip 0.1–0.2 mm i.d.) filled with Grace's insect cell culture medium (Invitrogen Corp., Carlsbad, CA, USA) for electric conductivity. Silver wires were inserted into the electrodes through acrylic holder at one end and were connected to a Syntech (Hilversum, the Netherlands) EAG probe through an interchangeable insert at the other. The identities of EAD active compounds were verified by matching retention times with purified synthetic standards injected onto these three columns. An antennal EAD response to the identified compound was verified with 10 ng samples of purified synthetic standards dissolved with *n*-hexane, and averaged EAD activity was calculated from five individual responses to the standards.

#### GC – mass spectrometry (GC–MS)

The EAD active components were analyzed by GC–MS (HP6890 GC and HP 5973N MSD, Agilent Technology, Huston, USA) in EI mode at 70 eV. Ion source temperature of 230°C, Q pole temperature of 150°C and interface temperature of 250°C.

Samples were analyzed on a non-polar column (HP1-MS), a polar column (DB23) or a chiral separation column (CP-Chirasil-DEX CB), under the same conditions as those of GC–EAD analyses, except for initial temperature (35°C) and initial holding time (3 min) in the case of the non-polar column. The GC–EAD active components were identified by mass spectral matches to library spectra (Wiley7n: Agilent Technology) followed by comparison of retention times with those of authentic compounds.

#### Field trap tests

Field trap tests in 2004 were performed using traps with cross barriers (30 cm width × 20 cm in height) and water basins (20 cm i.d. × 14 cm in depth) (Sankei Chemical Co. Ltd., Tokyo, Japan). Lures for the traps were made from plastic cups (5.8 cm i.d. × 2 cm in depth) containing cotton wicks infused with

1 ml of synthetic racemic quercivorol I (quercivorol), 50 ml of 70% ethyl alcohol solution (ethanol) or 50 ml of distilled water (control). Three kinds of traps were baited with racemic quercivorol I, ethanol and control and were set at a height of 1.5 m on trees in forests damaged by *P. quercivorus* at two sites in Kushibiki, Yamagata prefecture and at two sites in Keihoku, Kyoto prefecture, Japan in the summer, 2004. The captured beetles at each trap were collected every 4 days, during 28 days of tested period and counted for each sex. Lures were replenished and traps were shifted in each site every 8 days. In summer of 2005, we tested the effect of dose of synthetic racemic quercivorol, using same type of traps and lures with 20, 60, 200 or 600 µl of racemic quercivorol I in damaged forest in Atsumi, Yamagata prefecture (2005). In summer of 2006 we also tested attractance of optical isomers of quercivorol using funnel traps (Pherotech Inc., Delta, BC, Canada) and with 20 µl of purified enantiomers ((1S,4R)-isomer or (1R,4S)-isomer) or racemic quercivorol II in damaged forest in Oguni, Yamagata prefecture (2006). The captured beetles at each trap were collected every week and counted for each sex.

#### Statistical analysis for field trap test.

Statistical analyses were performed with SYSTAT ver. 9.01 (SPSS Inc., 1998). In all analyses a Type I error ( $\alpha$ ) rate of 0.05 was used. First, field data of numbers of captured beetles at each trap of Atsumi and Keihoku in 2004 were analyzed in three-way ANOVAs with the lure treatment, test site and period of the experiment as fixed factors. Because numbers of the beetles were heteroscedastic,  $\log_{10}(N+1)$ -transformations were performed prior to ANOVA analysis. There were few captured beetles in one site in Keihoku, therefore we also performed the same statistical analysis with this site excluded from the dataset. In cases where no significant interactions between factors occurred in ANOVA, pairwise differences between factor combinations were tested with the Bonferroni adjusting method (SPSS Inc., 1998). We also assessed effective dose of racemic quercivorol I in the data collected at Atsumi in 2005. Because there were no significant interactions between dose of racemic quercivorol and collection period, we performed ANCOVAs without such interactions. Last, we examined repeated measures ANOVAs for the experiment dealing with the optical isomers of quercivorol for the field test at Oguni in 2006.

To evaluate sex ratios for the captured beetles in response to racemic quercivorol I and ethanol, we conducted Fisher's exact probability tests for two-way tables.

## Results and Discussion

### *Analysis of Volatile component*

#### GC–EAD

Results of GC–EAD analyses are shown in Fig. 2. With

regards to boring frass volatiles from acetone extracts produced by unmated males, the antennae of both sexes responded to a peak A eluting at retention time 4.55 min and retention index (RI: 1123) on the non-polar column, 6.82 min (RI: 1577) on the polar column, and ca.10.0 min (RI: 1320) on the chiral column. EAD response to peak A was  $0.29 \pm 0.16$  mV (mean  $\pm$  SD) for female antennae of Fukushima,  $0.26 \pm 0.19$  mV for female antennae of Kyoto,  $0.34 \pm 0.32$  mV for male antennae of Fukushima, and  $0.43 \pm 0.25$  mV for male antennae of Kyoto. Neither were there no significant differences (t-test,  $P < 0.05$ ) of EAD responses to peak A between the two geographical locations, nor there were between males and females. However, female antennal responses were more reproducible than male antennal responses. EAD responses of peak A in boring frass volatiles from SPME samples were also observed (data not shown). It is possible that female antennae have more sensitive sensillae to this component than male antennae. The results suggest that this component plays important roles as an aggregation pheromone, for male and female orientation, but there is a possibility that other undetected compounds may play role as kiromone for male orientation.

The mean amount of the peak A was  $106.04 \pm 30.72$  ng from male whole body hexane extracts from Kyoto. Interestingly, we also detected a minute amount (less than 1 ng) of peak A from female whole body hexane extracts from Aizu-wakamatu. Hereafter, it will be necessary to clarify whether the female also secretes this component actively.

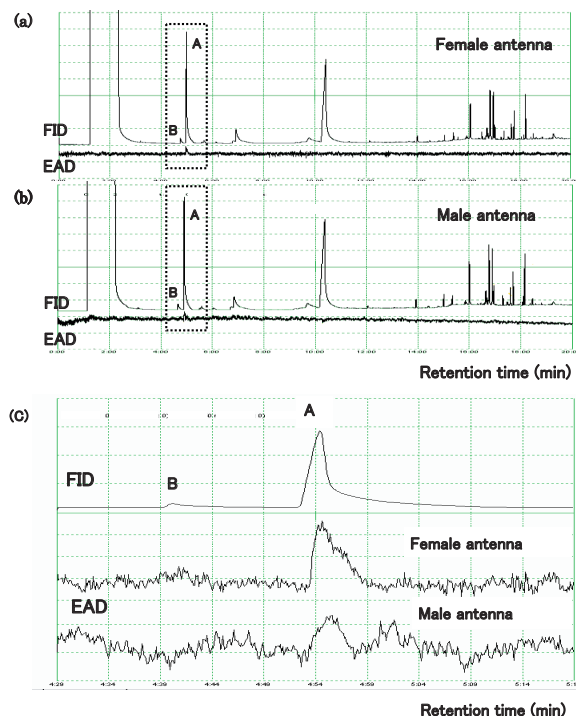


Fig. 2. Simultaneous recordings of the GC-FID (upper trace) and GC-EAD (lower trace) responses to the boring frass acetone extracts produced by unmated males of *P. quersivorus* using a non-polar column.

- (a) Female antenna from Kyoto  
 (b) Male antenna from Kyoto  
 (c) Magnification of the chromatograms of peaks A and B  
 A : EAD-active component B: stereo isomer of peak A

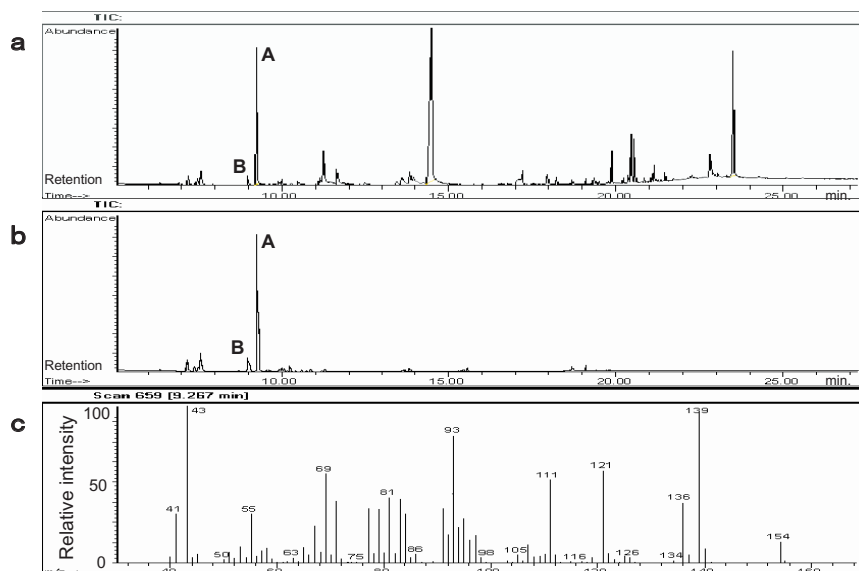


Fig. 3. Results of GC-MS analyses of boring frass volatiles extracted by acetone and SPME, using nonpolar column.

- (a) Total ion chromatogram of the boring frass volatiles from acetone extract.  
 (b) Total ion chromatogram of SPME sample.  
 (c) Mass spectrum of peak A from boring frass acetone extract.

A: EAD-active component B: *trans-stereoisomer* of peak A compound

Note that retention times for peaks A (9.27 min) and B (8.95 min) in (a) and (b) were identical, respectively.

## GC-MS

Results of GC-MS analyses of the EAD-active component (peak A: Fig. 2a, b) are shown in Fig. 3.

The EI mass spectrum of the peak A compound showed a molecular ion at  $m/z$  154 ( $M^+$ ) and characteristic fragment ion peaks at  $m/z$  43 ( $C_3H_7$ : base peak), 55, 69, 81, 93 ( $M-43-H_2O$ ), 111 ( $M-43$ ), 121 ( $M-CH_3-H_2O$ ), 136 ( $M-H_2O$ ), and 139 ( $M-CH_3$ ) (Fig. 3c). The mass spectral matching to library spectra of authentic compounds and comparable retention times with among peak A and authentic compounds indicated that the active peak was monoterpene alcohol of molecular weight 154 with a cyclohexene ring, and that the ring had a hydroxyl function on the 1<sup>st</sup> carbon and an isopropyl function on the 4<sup>th</sup> carbon. We determined the chemical structure of the EAD-active compound to be *cis-p*-menth-2-en-1-ol. Peak B also showed a molecular ion at  $m/z$  154 ( $M^+$ ) and similar characteristic fragment ion peaks with peak A, so we determined the compound to be a *trans-p*-menth-2-en-1-ol by comparison of authentic compounds for retention times and mass spectra. However, no reproducible response to peak B was detected by GC-EAD analyses.

Chiral analysis with the authentic compounds by Kashiwagi *et al.* (2006) indicates peak A to be (1S,4R)-*p*-menth-2-en-1-ol (Fig. 4), for which we proposed the name quercivorol after *P. quercivorus*.

## Field trap tests

Results of field trap test at Yamagata and Kyoto in 2004 were shown in Fig. 5. Number of captured beetles by quercivorol **I** and ethanol did not significantly differ (Fisher's exact probability test) among female and males, the numbers of both sexes were combined further statistical analyses. Because quercivorol traps attracted both sexes, quercivorol apparently acts as an aggregation pheromone, which has been reported in other *Platypus* (Renwick *et al.*, 1977, Shore and Mclean, 1983) rather than as a sex pheromone (Audino, *et al.*, 2005), such as sulcatol and sulcatone for *P. mutatus*. Results of the field trap test in 2004 showed that racemic quercivorol **I** traps attracted more beetles than ethanol traps or control traps, as shown in the three-way ANOVAs and the post-hoc test (Table 1, 2). The effects of lure and test sites were highly significant. In addition, test periods and lure  $\times$  site interactions were nearly significant using all test site data (Table 1a). Using the three sites where we were able to collect enough numbers of beetles, only the effect of lure was significant and all interactions between factors were not significant (Table 1b). We conducted a post-hoc test for each pair of lures with the Bonferroni adjusting method, and found a significant difference between racemic quercivorol **I** and other lures (Table 2).

On the results of field trap test at Atsumi Yamagata in

2005, the number of captured beetles significantly changed with dose of racemic quercivorol **I** (Fig. 6, ANCOVAs, dose of racemic quercivorol **I**:  $F_{1,7}=5.643$ ,  $P=0.049$ , test period:  $F_{1,7}=8.228$ ,  $P=0.024$ ). We suggest that the optimal dose of racemic quercivorol **I** is above 600  $\mu$ l / trap, though we cannot define exact value.

From the results of the field trap test at Oguni, Yamagata in 2006 using optical isomers, traps with both enantiomers and racemic quercivorol **II** caught the beetles, but in the control traps did not (Fig. 7). In total, we captured about twice as many beetles with (1S,4R) traps as with (1R,4S) and racemic quercivorol **II**. However, we cannot detect significant differences between lures (Repeated measured ANOVAs, type of lure:  $F_{3,16}=1.310$ ,  $P=0.306$ , test period:  $F_{1,16}=5.904$ ,  $P=0.027$ , type of lure  $\times$  test period:  $F_{3,16}=0.755$ ,  $P=0.535$ ). The captured number of beetles significantly changed between the test periods. The decreased number of beetles was likely due to weather changes between the test periods because the beetles' dispersal behavior is known to change in response to sunlight and wind (Ueda & Kobayashi, 2000).

Table 1. Results of a three-way ANOVA of the effects of lure (quercivorol\*, ethanol and water), test period and test site on log-transformed number of beetles collected per trap (a) for all sites, (b) for well-collected sites

\*racemic quercivorol **I**

(a)				
factor	df	MS	F-ratio	P
lure	2	1.789	35.876	< 0.0001
test site	3	0.439	8.799	0.001
test period	3	0.149	2.986	0.059
lure $\times$ test site	6	0.132	2.649	0.051
test site $\times$ test period	9	0.061	1.215	0.345
test period $\times$ lure	6	0.062	1.240	0.332
	18	0.050		
(b)				
factor	df	MS	F-ratio	P
lure	2	1.878	33.161	< 0.0001
test site	2	0.048	0.848	0.452
test period	3	0.172	3.039	0.071
lure $\times$ test site	4	0.105	1.860	0.182
test site $\times$ test period	6	0.073	1.285	0.334
test period $\times$ lure	6	0.085	1.503	0.258
	12	0.057		

Table 2. F-ratio (df =1, 22) (above diagonal) and P-value (below diagonal) for each pairwise comparison between lure type with the Bonferroni adjusting method. Number of beetles at two sites at Yamagata and one site at Kyoto were used.

	quercivorol	ethanol	water
quercivorol*	-	19.337	49.917
ethanol	0.0003	-	3.469
control	<0.0001	0.1582	-

\*racemic quercivorol **I**

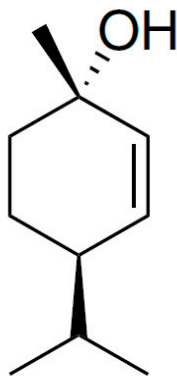


Fig. 4. Chemical structure of aggregation pheromone of *P. quercivorus*. (1*S*,4*R*)-*p*-menth-2-en-1-ol (quercivorol)

During 2006 of test period, we had much rain in spite of field trapping at the test sites in Yamagata, the captured numbers of the beetles by racemic quercivorol II in 2006 was much less than that by racemic quercivorol I in 2004. Because of their low density, we were not able to capture sufficient beetles for statistical analyses. However, (1*S*,4*R*)-isomer showed the most luring ability in the field. It showed concordance with electrophysiological activity to (1*S*,4*R*)-isomer of GC-EAD analysis, and we suggest that the (1*S*,4*R*)-isomer, quercivorol is the main component of the aggregation pheromone. Quercivorol is confirmed as novel pheromone component in insects (Witzgall *et al.*, 2004). Quercivorol was found in several kinds of plant leaves as an essential oil (Tabanca, *et al.*, 2001; Ciccio *et al.*, 2002; Albuquerque, *et al.*, 2004; Sadyrbekov *et al.*, 2006). To develop methods for mass trapping (Lei & Bakke, 1981) and communication disruption (Payne, 1981) against damage to oak forests by *P. quercivorus*, we need further analyses for the

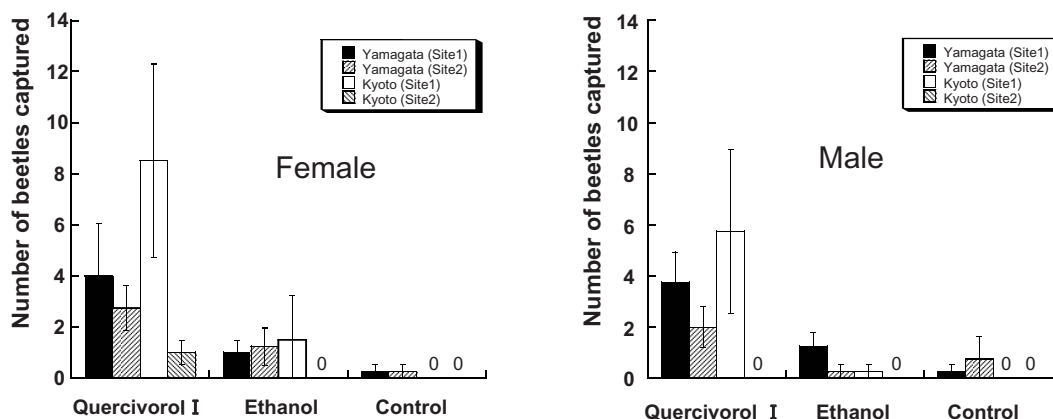


Fig. 5. Results of field trap tests at four sites in Yamagata (Kushibiki) and Kyoto (Keihoku) in 2004. Number of females (mean  $\pm$  SE) and males captured by traps with lures per 4 days during test period of 28 days. For details, see text and tables 1 and 2.

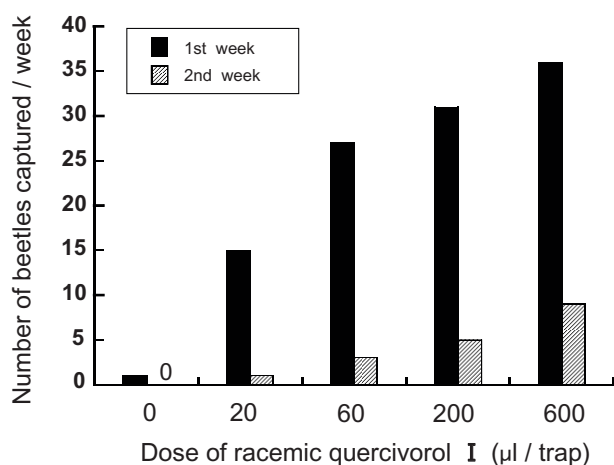


Fig. 6. Results of field trap tests at Yamagata (Atsumi) in 2005. Number of female and male beetles captured by traps with different doses per week during two weeks. For details, see text.

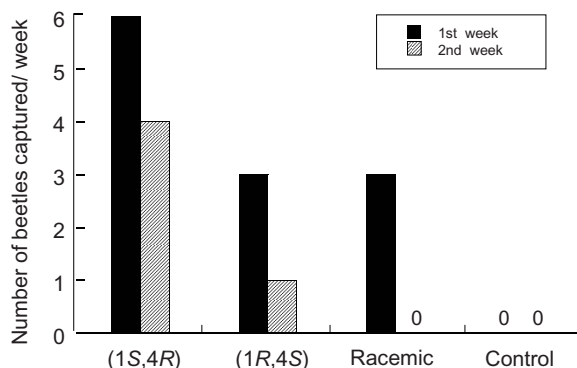


Fig. 7. Results of field trap tests at Yamagata (Oguni) in 2006. Number of beetles captured by traps with lures per week during two weeks of test period.  
 (1*S*,4*R*) : 20 µl of (1*S*,4*R*)-isomer (quercivorol)  
 (1*R*,4*S*) : 20 µl of (1*R*,4*S*)-isomer  
 Racemic: racemic quercivorol II (79.8 %, contains 4.1 % of *trans*-isomer)  
 Control : 50 ml of distilled water.

sub-component of the pheromone and combined effect of the pheromone and host kiromone component.

#### Acknowledgment

We appreciate the help of Ms. Ai Nozaki (Kyoto Prefectural Forestry Experimental Station) who supplied insects and collected boring frass. We thank Mr. Toshio Arihara (Fukushima Forest Research Center) for his assistance with collecting logs. Sincere gratitude is also extended to Drs. Kiyoshi Nakamuta and Richard P. Shefferson (Forestry and Forest Products Research Institute), for their critical reading of the manuscript and valuable advice. This work was supported in part by a Grant-in-Aid "Research Project for Utilizing Advanced Technologies in Agriculture, Forestry and Fisheries" from the Research Council, Ministry of Agriculture, Forestry and Fisheries of Japan, grant No.1775.

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## 養菌性キクイムシ、カシノナガキクイムシ（鞘翅目：ナガキクイムシ科） の集合フェロモン、(1*S*,4*R*)-*p*-メント-2-エン-1-オール

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### 要 旨

カシノナガキクイムシの集合フェロモンを、未交尾雄が穿入口付近に付着させた初期フラスの揮発性成分から、ガスクロマトグラフ触角電図法により分析した。ガスクロマトグラフ質量分析計による分析等の結果、ガスクロマトグラフ触角電図法により活性のあった成分の化学構造は (1*S*,4*R*)-*p*-メント-2-エン-1-オール（ケルキボロール）と決定された。ケルキボロールとその光学異性体を用いた野外試験の結果、この成分が集合フェロモンであることが証明された。

キーワード：カシノナガキクイムシ、集合フェロモン、(1*S*,4*R*)-*p*-メント-2-エン-1-オール、ケルキボロール、養菌性キクイムシ

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