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Aggregation pheromone of the Oriental spruce engraver *Pseudips orientalis*

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Abstract

1 Volatiles from the hindgut extracts of males of the Oriental spruce engraver *Pseudips orientalis* (Wood & Yin) (Coleoptera: Curculionidae, Scolytinae) of different phases of gallery development were analyzed by gas chromatography-mass spectrometry-flame ionization detection (GC-MS/FID) with both polar and enantioselective columns.

2 GC-MS/FID analyses showed that unmated males or males mated with one female produced approximately 95%-(−)-ipsenol and (−)-*cis*-verbenol as major components, as well as (−)-*trans*-verbenol, myrtenol, approximately 70%-(+)-ipsdienol and (−)-verbenone as minor or trace components. The release of these male-produced compounds was confirmed by GC analysis of an aeration sample of a *P. orientalis*-infested spruce log. Mating reduced production of the male-specific hindgut volatiles.

3 A field-trapping bioassay in Qinghai, China, showed that a ternary blend containing two major components, 97%-(−)-ipsenol (i.e. close to naturally produced enantiomeric ratio) and (−)-*cis*-verbenol, plus a minor component (−)-*trans*-verbenol, caught significantly more *P. orientalis* beetles (♂ : ♀ = 1 : 2.7) compared with the unbaited control. Subtraction of (−)-*trans*-verbenol from the active ternary blend had no significant effect on trap catches. The addition of (±)-ipsdienol (at 0.2 mg/day release) to the active ternary or binary blends significantly interrupted their trap catches. Replacing 97%-(−)-ipsenol with (±)-ipsenol in the ternary blend significantly reduced trap catches to a level that was no different from the blank control.

4 *Pseudips* species were sister to all other Ipini genera in a phylogeny reconstructed with mitochondrial cytochrome oxidase I DNA data for 51 Ipini and outgroup species.

5 The results obtained suggest that the two major components, 95%-(−)-ipsenol and (−)-*cis*-verbenol (at approximately 4–5 : 1), produced by unmated fed males, are probably the primary aggregation pheromone components for *P. orientalis*. In light of the phylogeny, the use of terpenoid semiochemicals as pheromones probably occurred early in the evolution of Ipini and these semiochemical blends were subsequently modified in the process of speciation.

Keywords *cis*-verbenol, Coleoptera, Curculionidae, enantiomeric composition, forest protection, GC-MS, ipsdienol, ipsenol, phylogeny, *Picea*, *Pseudips orientalis*, Scolytinae, semiochemical, *trans*-verbenol, trap.

Introduction

The Oriental spruce engraver *Pseudips orientalis* (Wood & Yin) (Coleoptera: Curculionidae, Scolytinae) was first

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described in 1986 as *Ips orientalis* Wood & Yin based on specimens collected from spruce trees in Xizang (Tibet) and Sichuan (China) during the 1970 s (Wood & Yin, 1986). It was, however, moved to a recently described genus *Pseudips* Cognato based on a phylogenetic analysis of 45 Ipini species using molecular [mitochomdrial DNA-cytochrome oxidase I (COI) sequence], morphological and behavioural characters (Cognato, 2000). *Pseudips orientalis* is related to two remaining North America members of the genus, *Pseudips concinnus* (Mannerheim) and *Pseudips mexicanus* (Hopkins), based on shared morphological and behavioural characters (Cognato, 2000). Recently, *P. orientalis* was recorded in several other western provinces of China, including Qinghai (Liu *et al*., 2007). *Pseudips orientalis* together with *Ips nitidus* Eggers and *Ips shangrila* Cognato and Sun are the most destructive forest beetles in the Maixiu Forest Park of Qinghai Province, China (Cognato & Sun, 2007), causing significant tree mortality both in plantations and in natural stands of the Qinghai spruce *Picea crassifolia* (Kom.) subsequent to 2001 (Xue *et al*., 2003; Liu *et al*., 2007, 2008). Data that detail its basic biology are limited to host colonization sequence (temporal and spatial niches) in relation to other sympatric bark beetle species (Liu *et al*., 2007).

As part of our research effort on the chemical communication systems of the three key spruce bark beetles native to western China, we recently identified the aggregation pheromone systems for *I. nitidus* (Zhang *et al*., 2009a) and *I. shangrila* (Zhang *et al*., 2009b). In the present study, we provide the first report on the identification of the aggregation pheromone system of *P. orientalis*, including (i) the potential pheromone composition; (ii) quantitative variations from different phases of gallery development; (iii) determination of the enantiomeric compositions of major chiral pheromone components; and (iv) the behavioural activity of the key male-specific compounds in the field in Qinghai, China. In addition, we sequenced part of the mtDNA COI for *P. orientalis* and discuss the phylogenetic relationship among *Pseudips* species and Ipini genera.

Materials and methods

Collection and preparation of samples

Field collections of live *P. orientalis* adult beetles of different gallery-development phases (phase 1, unmated males finishing nuptial chambers; phase 2, mated male with one female in the gallery; phase 3, mated male with two females in the gallery) were collected from naturally attacked windthrown spruce trees (*Picea crassiflia*) at Maixiu Forest Park, Huangnan Tibetan District, Qinghai Province, China, on 22–24 May 2008. Beetles from the same gallery were placed in a 2-mL polyethylene centrifuge tube (Fisher Scientific Co., Pittsburgh, Pennsylvania) and immediately put into a cooling box (approximately 4 °C). The centrifuge tubes were separated into categories of gallery-development phase on the day of collection, the hindguts were quickly dissected, and the sexes were separated carefully based on their elytral spine differences (Wood & Yin, 1986) and/or presence of the aedeagus or eggs. Guts from beetles of the same sex from the same gallery phase were transferred immediately to a glass vial with 1 mL of redistilled pentane, with 2 μg of heptyl acetate as internal standard.

Headspace volatiles from a freshly cut spruce log (diameter 12 cm, length 30 cm) with 20 *P. orientalis* males fed within pre-drilled holes for 24 h were sampled under field conditions using a battery operated pump coupled to a Reynolds® Oven Bag (48.2 × 59.6 cm; Reynolds, Richmond, Virginia) enclosure with two activated charcoal filter tubes in the air inlet on 24 May 2008. The volatiles released from the infested spruce log in the enclosure were trapped on a Porapak Q tube (50/80 mesh; 30 mg in Teflon tube: 3 mm × 35 mm) for 2 h (airflow 350 mL/min) and extracted with 1 mL of redistilled pentane. The same aeration and extraction procedures were used to collect volatiles from an uninfested spruce log of the same dimension and taken from the same tree.

Both hindgut and aeration extracts were shipped to the U.S.A. by express mail and maintained at −20 °C until they were analyzed chemically.

Chemical analysis

All *P. orientalis* hindgut and aeration samples were analyzed on a combined Agilent 6890N gas chromatograph (GC) and an Agilent 5973N mass selective detector equipped with a polar column (INNOWax; 60 m × 0.25 mm × 0.5 μm film thickness; Agilent Technologies, Wilmington, Delaware). The GC condition and temperature programme were the same as described by Zhang *et al*. (2009a) for *I. nitidus* and in Zhang *et al*. (2009b) for *I. shangrila* samples. Compounds were identified by comparison of retention times and mass spectra with those of authentic standards (see below).

GC-FID analysis

The hindgut samples were also injected into a Varian CP-3800 GC (Varian Inc., Palo Alto, California) equipped with a polar column (INNOWax; 30 m × 0.53 mm × 1.0 μm film thickness; Agilent Technologies) and flame ionization detection (FID) for compound quantification based on the internal standard (2 μg of heptyl acetate in each sample; assuming similar or identical response factors between the analytes and the internal standard). The GC condition and temperature programme used were as described by Zhang *et al*. (2009a, b).

Enantioselective GC-FID

The enantiomeric analyses of male hindgut extracts (from phases 1 to 2), and a synthetic mixture of several key *Ips* pheromone compounds including (±)-ipsenol, (±)-ipsdienol, (1*S*,2*S*)-(−)-*cis*-verbenol, (1*S*,2*R*)-(−)-*trans*-verbenol, amitinol, (1*S*)-(−)-verbenone, and *E*-myrcenol (50 ng/μL each in hexane) were conducted by injecting the samples splitless on a Varian CP-3800 GC equipped with an Rt-bDEXm™ column (30 m × 0.25 mm × 0.25 μm film thickness; Restek, Bellefonte, Pennsylvania). Helium was used as the carrier gas, and the injector/detector temperatures were both 230 °C. Column temperature was 80 °C for 1 min and rose to 200 °C at

2 °C/min. Elution orders of the (−)- or (+)-enantiomers of ipsenol and ipsdienol [(−)-eluted before (+)- for both compounds] were determined by injecting SPME (CAR/PDMS, 75 μm; Supelco, Bellefonte, Pennsylvania) samples of synthetic 97%-(+)-ipsdienol and 97%-(−)-ipsenol onto the same column. Because no pure (+)-enantiomers of *cis*- or *trans*-verbenol, and verbenone were available for comparison, their proposed enantiomeric assignments were based entirely on the retention time matches to the synthetic (−)-enantiomers.

Chemical standards

Synthetic compounds were obtained from various commercial and noncommercial sources: (±)-ipsenol [95%, chemical purity (cp)], (±)-ipsdienol (95% cp), (−)-*cis*-verbenol [98% cp, unknown enantiomeric purity (ep)] and (−)-verbenone (99% cp, unknown ep) (Bedoukian Research Inc., Danbury, Connecticut); amitinol (98% cp; W. Francke, Universität Hamburg, Germany); *E*-myrcenol (95.2% cp; SciTech, Czech Republic); 2-methyl-3-buten-2-ol (97% cp; Acros, Morris Plains, New Jersey); (−)-ipsenol (95% cp; 97% ep), (+)-ipsdienol (95% cp; 97% ep), (−)-ipsdienol (95% cp; 97% ep) and (−)-*trans*-verbenol (>95% cp, unknown ep) [Pherotech (Contech) International, Inc., Canada]; heptyl acetate (>98% cp, food grade) (Sigma-Aldrich, St Louis, Missouri).

Field trapping

To test for the behavioural activity of the potential semiochemicals, a field-trapping experiment was carried out on 6–22 May 2009 at the Maixiu Forest Park, Qinghai, China. Four sets of cross-barrier type traps (Pherobio Technology Co., Ltd, China) were set up along the edges of *P. crassifolia* forest stands on northern slopes at Qiliangou, Langnaihai, Longzanggou and Douheyan, respectively, with approximately 10 m between traps within each set, and >10 m from the nearest trees. Within each set, eight traps were baited with different blends (full or partial blends; racemic or enantiomerically pure) of the key male-produced volatile compounds in their natural production ratios; a ninth trap was left unbaited as a negative control. Each tested compound was released separately from a polyethylene bag (with or without fabric substrate felt for holding the chemicals) with different sizes and plastic film thickness. The positions of traps together with dispensers within each set were assigned randomly.

Phylogenetic analysis

DNA was extracted from *P. orientalis* individuals and approximately 800 bp from the 3′ end of the COI mitochondrial gene was amplified via polymerase chain reaction in accordance with the protocols described by Cognato and Sperling (2000). Both strands of DNA were sequenced, edited, and the resulting consensus sequence (GenBank accession number GU811707) was used in a Bayesian analysis including all known *Pseudips* species and 49 previously published COI sequences representing related genera *Ips*, *Orthotomicus*, *Pityogenes*, *Pityokteines*, [for Genbank accession numbers, see Cognato and Sun (2007)] and two outgroup species *Xyleborus spenos* and *Xylosandrus mancus* (Genbank accession numbers AF187142 and AF187143, respectively). Given that third position nucleotide substitutions are saturated for COI among Ipini species (Cognato & Sperling, 2000), we analyzed the sequences under maximum likelihood using a Bayesian estimation of the phylogeny. The analysis using MrBayes, version 3.1.2 (Hulsenbeck & Ronquist, 2001) included the data partitioned by codon position, a general time reversible with discrete gamma categories and the inclusion of the proportion of invariant sites model, flat priors, two runs with four Markov chains and 10 million generations, with trees sampled every tenth generation. Posterior probabilities were calculated with a majority-rule consensus of 750 000 trees.

Statistical analysis

Trap catch data were converted to proportion (P) of total captured beetles within each replicate. Data were then transformed by $\arcsin\sqrt{P}$ to meet the assumptions of normality and homogeneity of variances for analysis of variance (ANOVA). Means were compared by ANOVA followed by the Ryan–Einot–Gabriel–Welsh multiple Q test (SPSS 16.0 for Windows; SPSS Inc., Chicago, Illinois) at $\alpha = 0.05$ (Day & Quinn, 1989).

Results

Chemical analysis

Representative gas chromatograms of the male hindgut extracts from different gallery-development phases are shown in Fig. 1(A–C). These volatiles include ipsenol and *cis*-verbenol

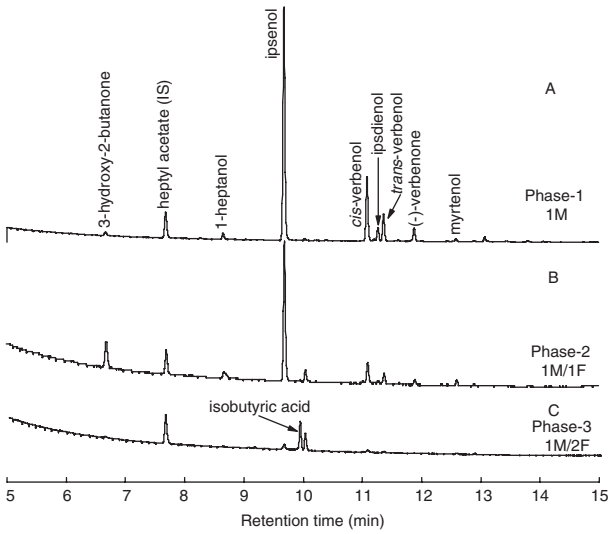

Figure 1 Representative gas chromatograms (polar column with flame ionization detector) of compounds in hindgut extracts of male *Pseudips orientalis* from different attack phases (A–C). Heptyl acetate (2 μg/sample) was added as an internal standard to the hindgut extracts. M, male; F, female.

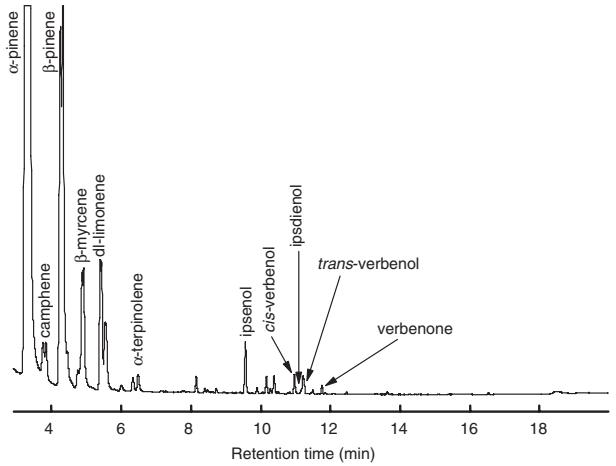

Figure 2 Representative gas chromatogram (polar column with flame ionization detector) of the aeration extract of a spruce log infested by 20 *Pseudips orientalis* males for >24 h. Beetle-produced volatiles are highlighted in bold.

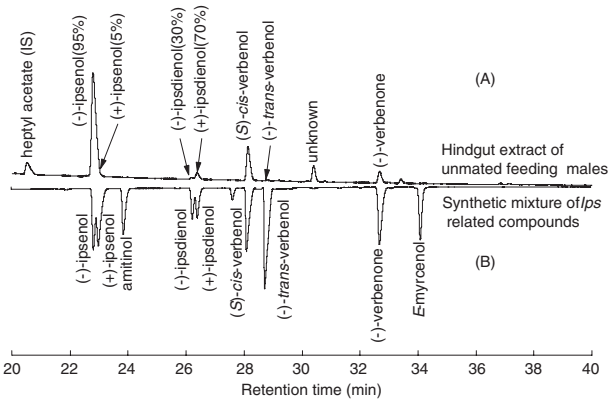

Figure 3 Enantioselective gas chromatography-flame ionization detection analyses (Rt-bDEXm™ column) of (A) compounds in a hindgut extract of unpaired male *Pseudips orientalis* from attack phase 1 and (B) a synthetic mixture of *Ips*-related compounds, including (±)-ipsenol, (±)-ipsdienol, amitinol, (−)-*cis*-verbenol, (−)-*trans*-verbenol, (−)-verbenone) and (*E*)-myrcenol (approximately 50 ng each per compound). Amitinol and *E*-myrcenol are achiral. Heptyl acetate (2 μg/sample) was added as an internal standard to the hindgut extract.

as major components, and *trans*-verbenol, ipsdienol, myrtenol and verbenone as minor ones (Fig. 1). 3-Hydroxy-2-butanone and 1-heptanol, commonly found in insect tissues, exist in all the samples (Fig. 1).

The amounts of ipsenol, *cis*-verbenol and *trans*-verbenol from phase 1 were estimated at 1450, 400 and 170 ng/male, respectively, whereas ipsdienol and verbenone were present at approximately 80 ng/male each. The amounts of ipsenol/*cis*-verbenol/*trans*-verbenol decreased to 325/56/28 ng/male at phase 2, and approximately 43/24/20 ng/male at phase 3, as the number of females admitted to the gallery increased (Fig. 1). No obvious *Ips* related aggregation pheromone components were detected in the female hindgut extract (data not shown). However, 3-hydroxy-2-butanone and 1-heptanol were detected in the female extracts at the same levels as found in the male extracts.

GC-mass spectrometry (MS)/FID analysis of an aeration sample of the *P. orientalis*-infested spruce log showed the release of male-produced compounds, ipsenol, *cis*-verbenol, *trans*-verbenol, ipsdienol and verbenone (Fig. 2), in addition to host monoterpenes (including two major components, α-pinene and β-pinene, accounting for >70% total volatiles) and other conifer-related compounds. These beetle-produced compounds were not detected from the aeration sample of an un-attacked spruce log (data not shown).

GC-FID analysis with an enantioselective stationary phase of the hindgut extracts of *P. orientalis* males from phases 1–2 and a synthetic mixture of *Ips* pheromone compounds showed that *P. orientalis* males produced 95%-(−)-ipsenol and 70%-(+)-ipsdienol (Fig. 3). The predominant enantiomers of other chiral compounds were tentatively determined as (−)-*cis*-/(−)-*trans*-verbenol and (−)-verbenone (Fig. 3).

Field-trapping experiment

Because *P. orientalis* populations were low and it rained continuously during the short spring flight, only four replicates were achieved in the field-trapping experiment (early to mid-May, 2009) with a total catch of 461 beetles. There were only two blends showing significantly higher trap catches compared with the unbaited blank traps: a ternary blend containing two major components, 97%-(−)-ipsenol (close to naturally produced enantiomeric composition) and (−)-*cis*-verbenol, plus a minor component *trans*-verbenol, and a binary blend of 97%-(−)-ipsenol and (−)-*cis*-verbenol (Fig. 4). The ternary blend appeared to catch more *P. orientalis* beetles compared with the binary one, although the results were not statistically different. Addition of (±)-ipsdienol (at 0.2 mg/day release) to the active ternary or binary blends significantly reduced their trap catches (Fig. 4). Replacing 97%-(−)-ipsenol with the inexpensive (±)-ipsenol in the ternary blend significantly reduced trap catches to a level that was no different from the blank control (Fig. 4); however, such replacement in the binary blend did not show significant reduction in trap catches. The sex ratio of captured beetles was estimated as 1 : 2.7 (♂/♀) based on the pooled sample.

Phylogenetic analysis

All parameters of the Bayesian analysis reached stability within 1.5 million generations and parameters between runs did not vary (average standard deviation of spilt frequencies, $p = 0.009$). *Pseudips* species are sister to the Ipini genera, although this analysis suggests that the placement of *P. orientalis* is unresolved with respect to its relationship to the clade of North American *Pseudips* species [*P. concinnus* (Mannerheim) and *P. mexicanus* (Hopkins)] and to the clade of the remaining Ipini species (Fig. 5). The other Ipini genera were monophyletic with high posterior probabilities (>90%). The relationships of the other Ipini genera and species are similar to the results found with other DNA data and phylogenetic analyses, with the exception of a monophyletic *Orthotomicus* (Cognato, 2000; Cognato & Sun, 2007; Jordal *et al.*, 2008).

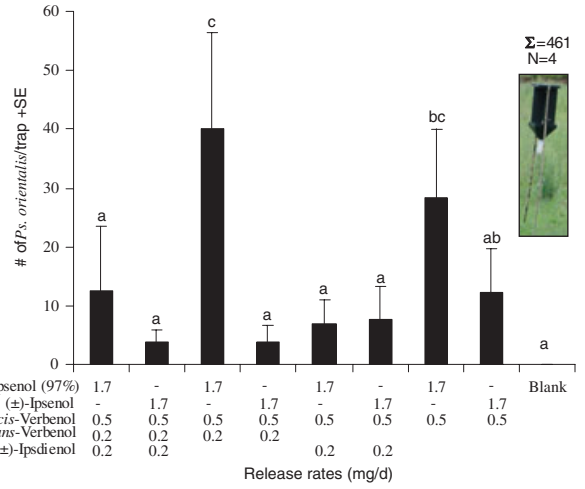

Figure 4 Mean captures ($N = 4$) of *Pseudips orientalis* in cross-barrier traps (photo inset) baited with different combinations of the key male-produced volatile compounds in their natural production ratios on 6–22 May 2009, Maixiu Forest Park, Qinghai, China. An unbaited trap served as the negative control. Bars with the same letter above are not significantly different ($P > 0.05$) by Ryan–Einot–Gabriel–Welsh (REGW) multiple Q test after analysis of variance on the arcsin$\sqrt{P}$ transformed data of the relative catches [i.e. proportion (P) of total captured beetles within each replicate]. Each tested compound was released separately from a polyethylene (PE) bag dispenser. 97%-(−)- or (±)-ipsenol: 50 mg in a 12-mil (approximately 0.305 mm) PE-bag (1.4 × 5.0 cm; with a 1.0 × 4.5 cm felt); (−)-*cis*-verbenol: 60 mg in a 4-mil (approximately 0.10 mm) PE-bag (3.0 × 5.0 cm, without felt); (−)-*trans*-verbenol: 20 mg in a 12-mil (approximately 0.305 mm) PE-bag (1.8 × 5.0 cm; with a 1.0 × 4.5 cm felt); and (±)-ipsdienol: 15 mg in a 12-mil (approximately 0.305 mm) PE-bag (1.2 × 2.5 cm; with a 0.5 × 2.0 cm felt). The release rates were measured in a laboratory hood at 21–22 °C for 7–10 days.

Discussion

This is the first report on the chemical and behavioural analysis of the aggregation pheromone system of the Oriental spruce engraver *P. orientalis*. The GC-MS/FID analyses clearly showed that unmated males (after completing the nuptial chambers; phase 1) or males mated with one female (phase 2) produced ipsenol and *cis*-verbenol as major components, and *trans*-verbenol, myrtenol, ipsdienol and verbenone as minor or trace components (Fig. 1). The release of these male-produced compounds was confirmed by the aeration sample of a *P. orientalis*-infested spruce log (Fig. 2). Similar to *I. nitidus* and *I. shangrila*, 3-hydroxy-2-butanone and 1-heptanol, commonly found in insect fat body (Zhang *et al*., 2006), were also present in all male and female hindgut extract samples of *P. orientalis*, and are presumably not part of the male-produced aggregation pheromone system (Zhang *et al*., 2009a). Mated females showed little or trace amounts of these semiochemicals (data not shown), in agreement with previous studies indicating that female *Ips* or its close relatives do not produce behaviourally relevant amounts of aggregation pheromones during attacks (Wood, 1982; Byers, 1989b).

GC-FID analyses indicated an obvious trend in the quantities of male-produced volatiles during different gallery-development phases. As with many other *Ips* spp. (e.g. *I. nitidus*, *Ips typographus* and *Ips subelongatus*), the maximum production of the major components of *P. orientalis* occurred in phase 1, when the nuptial chamber was finished by the unmated male (Fig. 1A) (Birgersson *et al*., 1984; Zhang *et al*., 2000, 2009a). Subsequently, mating reduced the production of the male-specific major hindgut volatiles. After being mated with two females (harem size = 2; phase 3), males released only trace to tiny amounts of male-specific compounds, suggesting that males in this phase may not be capable of attracting females or other males (Fig. 1C).

Enantioselective GC analysis indicated that *P. orientalis* males produced approximately 95%-(−)-ipsenol as the dominant component (Fig. 3). This biased (−)-enantiomeric composition of ipsenol is very common in the genus *Ips*; indeed, production of >90%-(−)-ipsenol as a part of aggregation pheromone systems (or major male-specific volatile compounds) has been reported in many Ipini species, such as *Ips acuminatus* (Bakke, 1978; Kohnle *et al*., 1988), *Ips cembrae* (Francke & Vite, 1983), *Ips cribricollis* (Eichh.) (Francke *et al*., 1986), *Ips grandicollis* (Vité *et al*., 1976), *Ips latidens* (Miller *et al*., 1991), *Ips mannsfeldi* [now recognized as *Orthotomicus latidens and Orthotomicus mannsfeldi* by Cognato and Vogler (2001)] (Kohnle *et al*., 1993), *I. nitidus* (Zhang *et al*., 2009a), *Ips paraconfusus* (Silverstein *et al*., 1966; Francke *et al*., 1986), *Ips perturbatus* (Holsten *et al*., 2000), *Ips schmutzenhoferi* Holzschuh (Francke *et al*., 1988) and *I. subelongatus* (Zhang *et al*., 2000). The enantiomeric ratio of ipsdienol [approximately 70%-(+)], one of the minor components is unique for *P. orientalis* and is different from those reported for other Eurasian *Ips* spp. (Kohnle *et al*., 1988, 1991; Zhang *et al*., 2007), including its two sympatric and potentially competing species *I. nitidus* [74%-(−)] (Zhang *et al*., 2009a) and *I. shangrila* [99%-(+)] (Zhang *et al*., 2009b). This enantiomeric ratio, however, is similar to those of several eastern and northern populations of the North American *I. pini* [approximately 61%-(+)] (Seybold *et al*., 1995). Other chiral compounds were tentatively determined as (−)-*cis*- and (−)-*trans*-verbenol, and (−)-verbenone, based on the retention time comparison with the synthetic standards (Fig. 3), which are similar to the reported absolute configurations for many *Ips* bark beetles (Kohnle *et al*., 1988), including the sympatric *I. nitidus* (Zhang *et al*., 2009a) and *I. shangrila* (Zhang *et al*., 2009b).

The field-trapping data from the present study showed that a ternary blend containing two major components, 97%-(−)-ipsenol (close to the naturally produced enantiomeric ratio) and (−)-*cis*-verbenol, plus a minor component (−)-*trans*-verbenol, and a binary blend of the two major components [97%-(−)-ipsenol/(−)-*cis*-verbenol] caught significantly more *P. orientalis* beetles compared with the unbaited control (Fig. 4). Subtraction of (−)-*trans*-verbenol from the active ternary blend had no significant effect on trap catches. This compound was also detected in several other Eurasian *Ips* species as a minor component; for example, *I. nitidus* (Zhang *et al*., 2009a), *I. typographus* (Birgersson *et al*., 1984), and *Ips duplicatus* (Byers *et al*., 1990; Schlyter *et al*., 1992; Zhang *et al*., 2007), although it was not considered to be part of their aggregation pheromone systems (Schlyter *et al*., 1987). Replacing 97%-(−)-ipsenol with the inexpensive (±)-ipsenol

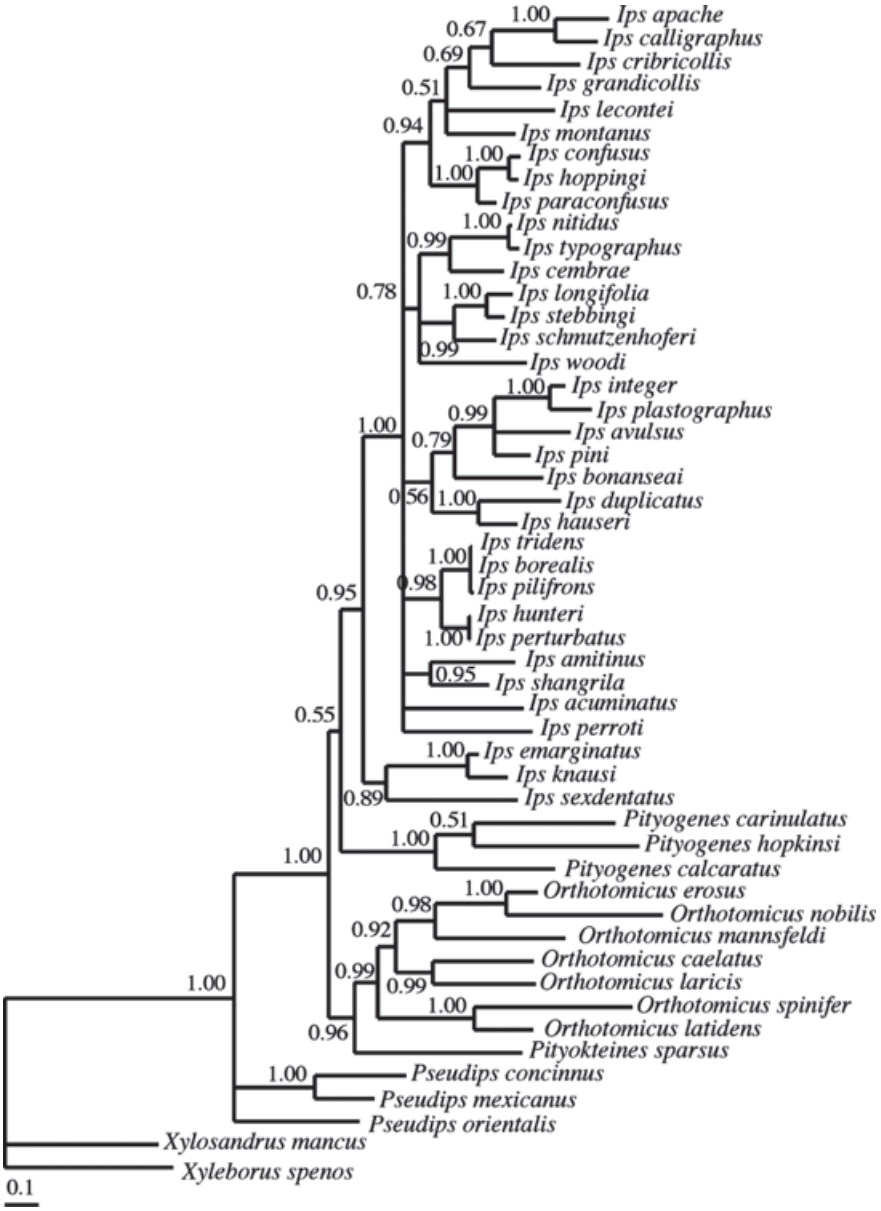

Figure 5 Majority rule consensus of 750 000 trees found with a Bayesian analysis of Ipini species. Numbers at branch nodes are posterior probabilities. For details of the analysis, see text.

in the ternary blend reduced trap catches to a level not significantly different from the blank control (Fig. 4), whereas such replacement in the binary blend did not show significant reduction in trap catches. The binary blend with (±)-ipsenol and (−)-*cis*-verbenol appeared to be weakly attractive, although it was not different from the unbaited control. Such marginal differences in trap catches may be a result of heterogeneity of variance among the treatments, resulting from the extremely low abundance and limited number of replicates obtained. More field-trapping experiments are needed to determine whether the natural ratio of ipsenol enantiomers will prove superior to racemic ipsenol in the binary blend, and, even more importantly, whether the inexpensive binary blend will be significantly more attractive than the unbaited control.

The phylogenetic analysis in the present study supports previous findings of the sister relationship of *Pseudips* to the other Ipini genera (Fig. 5) (Cognato, 2000; Cognato & Vogler, 2001; Jordal *et al*., 2008). Although additional DNA data and further analyses would potentially help resolve the placement of *P. orientalis*, inferences concerning pheromone evolution can be made. The use of terpenoid semiochemicals as pheromones probably occurred early in the evolution of Ipini bark beetles, followed by modifications of semiochemical blends in different species. The proposed two-component aggregation pheromone [95%-(−)-ipsenol/(−)-*cis*-verbenol] of *P. orientalis* is quite different from the pheromone systems reported for any other scolytine bark beetles, including *P. mexicanus.* According to Seybold (1992), montane *P. mexicanus*

produced both ipsdienol [90%-(−)] and ipsenol (enantiomeric composition not reported), whereas, in the coastal *P. mexicanus*, only 90%-(−)-ipsdienol was found. The status of these compounds as pheromone components for *P. mexicanus* has not been established, although it was reported that (−)-ipsenol alone was attractive (Miller *et al*., 1991; Savoie *et al*., 1998). Moreover, significantly more *P. mexicanus* beetles were captured in traps baited with (−)-ipsenol/(±)-ipsdienol (Savoie *et al*., 1998) in British Columbia, Canada, suggesting that both ipsenol and ipsdienol may contribute to the pheromone blend for this species, with ipsenol as the main component in this region. Such compositional disparity in the pheromone systems between *P. orientalis* and *P. mexicanus* is similar to the plasticity of pheromonal composition observed in other related Ipini species (Cognato *et al*., 1997). The data from the present study also supports the saltational evolution theory proposed by Symonds and Elgar (2004a) [i.e. within certain phylogenetic constraints, pheromone evolution in bark beetles is characterized by large saltational shifts, resulting in substantial phenotypic (i.e. pheromonal composition) differences between sibling species]. Unfortunately, nothing is known about the aggregation pheromone of the third species in the genus, *P. concinnus*; thus, the extent of semiochemical variability within this genus remains to be discovered.

Pseudips orientalis and two other sympatric *Ips* bark beetles, *I. nitidus* and *I. shangrila*, share similar spatial and temporal niches on *P. crassifolia* in Qinghai, China (Liu *et al*., 2007). To a certain extent, this niche similarity may result in interspecific competition and reproductive isolation pressures (Wood, 1982). *Ips nitidus* begins its dispersal flight for host-searching after emerging from the litter layer in early May in Qinghai. *Ips nitidus* normally attacks on wind-thrown, weakened, or sometimes even healthy, Qinghai spruce trees, mostly on the mid-lower portion of the trunk. Upon arrival at potentially suitable host trees (by random landing or primary attraction), males initiate attacks and produce aggregation pheromone, consisting of 2-methyl-3-buten-2-ol, 74%-(−)-ipsdienol, and (−)-*cis*-verbenol (Zhang *et al*., 2009a) (Table 1) to attract both conspecific males and females for mass-attacks. Approximately 1–2 weeks after the first attacks by *I. nitidus*, *I. shangrila* start their host-searching dispersal flights, and select the upper parts of suitable host trees for mass attacks by random landing, primary attraction and/or cross-attraction to *I. nitidus* aggregation pheromone (Zhang *et al*., 2009b) (Table 1). A weak cross-attraction to *I. nitidus* aggregation pheromone blend by *I. shangrila* was reported by Zhang *et al*. (2009b) (Table 1), which might benefit the latter to quickly find the suitable tree that has recently been successfully attacked or weakened by *I. nitidus* in the absence of its conspecific aggregation pheromone and has the upper portion of tree trunk available for attacks. After boring into the spruce bark and feeding, mostly on the upper part of the trunk, *I. shangrila* males produce and release their own aggregation pheromone [2-methyl-3-buten-2-ol, (+)-ipsdienol, and (−)-*cis*-verbenol] to attract the conspecific males and females for mating and aggregation (Zhang *et al*., 2009b) (Table 1). The chemical composition of the *I. shangrila* pheromone system is similar to that of *I. nitidus* (Zhang *et al*., 2009a), although the enantiomeric composition of the major component ipsdienol in *I. shangrila* [approximately 99%-(+)] (Zhang *et al*., 2009b) differs from that of the *I. nitidus* [74%-(−)] (Zhang *et al*., 2009a). No obvious cross-attraction of *I. nitidus* beetles to *I. shangrila* aggregation pheromone, however, was found, and the presence of *I. shangrila* aggregation pheromone did not appear to inhibit the attraction of *I. nitidus* to its conspecific pheromone (Q.-H. Zhang, unpublished data). A lack of mutual inhibition and evidence for at least a partial cross-attraction, such as found for *I. nitidus* and *I. shangrila*, is likely to occur wherever sympatric species of bark beetles commonly co-exist on the same host tree (Poland & Borden, 1994).

Pseudips orientalis, similar to *I. nitidus*, also attacks the mid-lower part of the spruce tree trunk, although its dispersal flight is normally 1–3 weeks earlier than that of *I. nitidus* or *I. shangrila* (F.-Z. Han, personal observation). Such partial temporal segregation among these sympatric bark beetle species may significantly reduce their interspecific competition. In addition to the partial temporal separations (and spatial segregation with *I. shangrila*), *P. orientalis* produces an aggregation pheromone blend [95%-(−)-ipsenol/(−)-*cis*-verbenol] that is quite different from *I. nitidus* (Zhang *et al*., 2009a) and *I. shangrila* (Zhang *et al*., 2009b) (Table 1). Ipsdienol, as one of the major pheromone components of *I. nitidus* [74%-(−)] and *I. shangrila* [99%-(+)], strongly inhibited the attraction of *P. orientalis* to its pheromone (Fig. 5 and Table 1). It is possible, although not yet demonstrated, that ipsenol, the major pheromone component of *P. orientalis*, may also inhibit the pheromone responses of its sympatric species, *I. nitidus* and *I. shangrila*. This could explain the rare co-occurrence of *P. orientalis* with *I. nitidus* or *I. shangrila* on the same infested spruce tree. The best known example for

Table 1 Male-produced semiochemicals of the three sympatric spruce bark beetles solely occurring in Western China

Species	Semiochemicals							
		Ipsdienol						
	2-Methyl-3-buten-2-ol	74%-(−)	50%-(−)	70%-(+)	99%-(+)	(−)-*cis*-verbenol	90%-(−)-ipsenol	(−)-*trans*-verbenol
Ips nitidus	♂ = (+)	♂ = (+)	♂ ≠ (+)	—	—	♂ = (+)	(−)?	♂ = (0)
Ips shangrila	♂ = (+)	♂ ≠ (+)	♂ ≠ (+)	—	♂ = (+)	♂ = (+)	(−)?	♂ = (−)
Pseudips orientalis	—	♂ ≠ (?)	♂ ≠ (−)	♂ = (?)	♂ ≠ (?)	♂ = (+)	♂ = (+)	♂ = (0)

♂ = (+): produced by the males as part of the aggregation pheromone blend; ♂ = (−): produced by the males but showing an inhibitory effect when added to its pheromone blend; ♂ = (0): produced by the males but showing no significant positive or negative effect when added to its pheromone blend; ♂ = (?): produced by males but behavioral functionality not yet tested; ♂ ≠ (+): not produced by males but attractive when combined with other pheromone components in blend; ♂ ≠ (−): not produced by males but inhibitory to its pheromone response; ♂ ≠ (?): likely inhibitory but not tested yet.

mutual inhibition of aggregation pheromone response among sympatric bark beetle species is the interspecific pheromonal interaction of *Ips pini* and *I. paraconfusus* competing in *Pinus ponderosa* in California (Birch & Wood, 1975). Nevertheless, mutual (or directional) inhibition of heterospecific pheromones suggests an agonistic relationship between species, and could be important in delineating breeding areas for sympatric bark beetles that normally do not co-infest the same host tree (Poland & Borden, 1994).

Moreover, a strong disparity in pheromone systems (i.e. discrimination among their pheromone blends) among the sympatric (i.e. competitive or cooperative) bark beetle species and their potential semiochemical interactions (such as mutual or directional inhibition; for details, see above) plays an important role in maintaining their mass-attack sequences (e.g. partial niche separation) and reproductive isolation, and regulating spatial and temporal competition (Wood, 1982; Byers, 1989a, b; Schlyter *et al*., 1992; Zhang *et al*., 2008, 2009a, b). Whether or not the sympatry or syntopy of different bark beetle species on the same host tree species has an effect on the evolution of their pheromone systems is questionable (Symonds & Elgar, 2004b).

The results obtained in the present study suggest that the two major components, 95%-(−)-ipsenol and (−)-*cis*-verbenol (at approximately 4–5 : 1), produced by unmated fed males, are probably the aggregation pheromone components for *P. orientalis*. Another semiochemical found in male hindgut tissue, (−)-*trans*-verbenol, may not be part of the aggregation pheromone system, although it merits further field bioassays to determine its behavioural function. More field-trapping experiments on optimal component ratios [especially using the inexpensive, racemic (±)-ipsenol], release rates, and dispenser technology are in progress. From a practical point of view, an optimized/simplified lure consisting of (±)-ipsenol and *cis*-verbenol (with optimal ratio and release rates) might have a commercial potential as a monitoring and mass-trapping tool in integrated pest management programme against this serious forest pest (Schlyter *et al*., 2001). The binary blend with enantiomerically pure (−)-ipsenol and *cis*-verbenol would be superior in performance, although might not prove economically viable at operational levels in the outbreak areas in China.

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