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HOST MICROBE INTERACTIONS

Gut-Associated Bacteria of *Dendroctonus valens* and their Involvement in Verbenone Production

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Abstract Bark beetles are the most important mortality agent in coniferous forests, and pheromones play important roles in their management. *Dendroctonus valens* LeConte was introduced from North America to China and has killed millions of healthy pines there. Trapping with semiochemicals and pheromones was deployed in *D. valens* management in the last decade, but little is known about the ability of gut bacteria to produce the pheromone. In this study, we analyzed the volatiles in *D. valens* guts and frass after antibiotic treatment versus control. Then, we isolated and identified the bacteria in *D. valens* guts and frass, examined verbenone (a multifunctional pheromone of *D. valens*) production by 16 gut bacterial isolates from the precursor *cis*-verbenol at three concentrations, and further compared the cytotoxicities between the *cis*-verbenol and verbenone to the bacterial isolates. *cis*-Verbenol was not detected in the frass in the control group, but it was in the antibiotic treatment. The amount of verbenone was significantly suppressed in *D. valens* guts after antibiotic treatment versus control. Thirteen out of 16 gut bacterial isolates were capable of *cis*-verbenol to verbenone conversion, and *cis*-verbenol had stronger cytotoxicities than verbenone to all tested gut bacterial isolates. The bacterial species capable of verbenone production largely exists in *D. valens* guts and frass, suggesting that gut-associated bacteria may help the bark beetle produce the pheromone verbenone in guts and frass. The bacteria may benefit from the conversion due to the reduced cytotoxicity from the precursor to the beetle pheromone.

Keywords Bark beetle · Gut bacteria · Pheromone production · *cis*-Verbenol · Verbenone

Introduction

Pheromones serve as important mediators of chemical communication for a variety of organisms [27], and insect pheromones are applied widely and play an important role in sustainable pest management strategies [20, 33, 68]. Insect gut bacteria have been reported to be involved in pheromone production and as kairomonal mediators of species interactions [13–15, 37, 52]. For example, gut microbiota of locusts produced components of aggregation pheromones in guts and in frass [13–15] and intestinal bacterial communities of termite *Reticulitermes speratus* produced colony-specific chemical cues that enabled nestmate recognition [37].

Bark beetles (Coleoptera: Curculionidae: Scolytinae) tend to be the most economically important pests of all coniferous forests, causing high levels of conifer mortality [20, 41, 46]. Their pheromones have been exploited to prevent attacks on

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living trees and to conduct area-wide mass trapping campaigns [50, 66, 68]. The pheromones of bark beetles include several oxygenated monoterpenoid compounds that are derived from the detoxification of the defensive monoterpenes of host conifers [3, 69]. α-Pinene, a prominent defensive monoterpene in conifers, is a precursor in the biosynthesis of verbenone, one of the common pheromones of bark beetles [3, 20, 63]. A variety of microorganisms may be involved in the production or conversion of pheromones. A strain of *Bacillus cereus* isolated from *Ips paraconfusus* guts was capable of producing the pheromone verbenol when exposed to α-pinene [6]. Yeasts associated with *I. paraconfusus* and *Dendroctonus ponderosae* and mycangial fungi from *Dendroctonus frontalis* converted verbenol to verbenone [5, 26, 32]. However, antibiotic-fed *I. paraconfusus* and axenically reared *D. ponderosae* and *I. paraconfusus* were able to produce verbenol in the presence of α-pinene [8, 10], which suggests that bark beetles can synthesize at least some pheromones independent of microbes. It remains to be answered to what extent the microorganisms associated with bark beetles are capable of synthesizing and metabolizing pheromones.

The red turpentine beetle (RTB), *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae), introduced from North America, has caused mortality of more than ten million healthy pines in central areas of Northern China [55]. The chemicals *cis*-verbenol, *trans*-verbenol, myrtenol, and verbenone are common gut volatiles in both sexes of *D. valens* in China [55], among which the verbenols and verbenone have been shown to be pheromones of some scolytine bark beetles [20, 23, 38]. *cis*-Verbenol was shown to inhibit the response of *D. valens* to attractant-baited traps in America when applied with racemic ipsenol and (+)-ipsdienol but did not exert any attractive or anti-attractive function in field trapping of *D. valens* in China [18, 73]. This gut volatile could be further converted to verbenone, which serves as an attractant to *D. valens* at low levels in China but as a repellent at high concentration both in North America and in China [19, 47, 72]. These chemicals have been successfully applied in *D. valens* control programs with other semiochemicals [44]. However, the role of gut bacteria in verbenone production has yet to be explored. In this study, we focused on the following questions: Are gut bacteria involved in verbenone production, and if so, then what species are capable of the biochemical transformation, and how abundant are they? Is the precursor of verbenone more toxic than verbenone to those bacteria? To answer these questions, we first analyzed volatiles in the guts and frass of adult *D. valens* after antibiotic treatment versus control and then isolated and identified the bacterial isolates from guts and frass of adult *D. valens* obtained from the field and subjected the *D. valens* gut isolates to further examination of their chemical conversion capacity. We then measured and compared the cytotoxicity of the chemicals to the bacterial isolates.

Materials and Methods

Insects and Media

Adult beetles were captured in newly attacked pine stumps in the Tunlanchuan Forestry Station (37° 48′ N, 111° 44′ E, average elevation 1400 m), Shanxi province, in June 2013. Two sampling sites within the station were chosen, Beishe Mountain and Laoyagou Mountain (about 13 km apart) in the station were chosen, and at each sampling site, 15 newly attacked pine stumps (at least 200 m apart) were randomly selected for beetle and frass sampling. To make phloem medium, *Pinus tabuliformis* phloem was freeze-dried, ground, and autoclaved to sterilize and remove volatile monoterpenes, as described by Wang et al. [64]. Ten grams of agar (NewProbe, China) was mixed with 300 ml boiled distilled water and 20 g ground phloem [64]; then, 30 mg α-pinene was added after cooling. About 6 ml of this phloem medium amended with α-pinene was then poured into each Petri dish (35-mm diameter and 15-mm height) and dried for 12 h. In the bacterial conversion experiment, M9 minimal salt medium (pH=7.4) containing 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, and 1 g NH_4Cl per liter was used [49]. Tryptic soy agar (TSA), tryptic soy broth (TSB), and Luria-Bertani medium were purchased from Sigma-Aldrich (Shanghai, China).

Chemicals

(+)-α-Pinene (≥99 % purity), (S)-*cis*-verbenol (95 % purity), (1S)-(-)-verbenone (94 % purity), and heptyl acetate (≥98 % purity) used for all experiments were purchased from Sigma-Aldrich (Shanghai, China). Authentic standards (1R)-(-)-myrtenol (95 % purity) and *trans*-verbenol (82 % (−), 94 % purity) used for identification of gut volatiles were obtained from Sigma-Aldrich and Phero Tech (Delta, British Columbia, Canada), respectively. Antibiotic streptomycin sulfate and ampicillin sodium salt were purchased from Sigma-Aldrich (Shanghai, China). Tetracyline HCl was obtained from Genview (Tianjing, China) and nystatin was obtained from Aladdin (Shanghai, China).

Volatiles in Gut and Frass after Different Treatments

Streptomycin sulfate, ampicillin sodium salt, tetracyline HCl, and nystatin were added into phloem medium described above to make antibiotic phloem medium with a final concentration of each antibiotic estimated to be 4.1 μg/μl [8]. Adult beetles (40♀, 40♂) were randomly chosen, surface-sterilized by immersion in 70 % ethanol for 5 s, washed with distilled water for 5 s, and then separated into two groups. After that, they were introduced into the medium individually. The beetles in phloem medium (20♀, 20♂) were set as the control group and those in antibiotic phloem medium (20♀, 20♂) were set as the

treatment group. After 96-h feeding, feeble beetles were discarded and 20 vigorous ones (5♀, 5♂ for each group) were selected and dissected individually as before [36] under sterile conditions. Then, each dissected gut and its frass were suspended in 200 μl of 10 % phosphate buffer saline solution (PBS), crushed, sonicated for 1 min, and vortexed at medium speed for 10 s, and the suspension was plated (dilution factors varied from 10^1 to 10^4) on TSA to count numbers of colonies (colony forming units (CFU)) [61]. Other beetle guts and frass in the control group (12♀, 11♂) and antibiotic treatment group (12♀, 11♂) were extracted individually with hexane-containing heptyl acetate as an internal standard [43], and the solutions were kept in 2-ml vials (Agilent, USA) and stored at −20 °C for chemical analysis.

Isolation, Identification, and Phylogenetic Analyses of Bacteria

Individual guts from adult beetles (n=119) and frass (n=40) collected were crushed in 200 μl of 10 % PBS, respectively, sonicated for 1 min, and vortexed at medium speed for 10 s, and the suspension was plated on TSA (dilution factors varied from 10^2 to 10^6). After incubation at 28 °C for 12–48 h, colonies from each sample were selected and streaked for purification. Pure cultures were morphologically categorized and counted by the combination of size, color, thickness, transparency, and texture. Then, sample isolates were selected for 16S rDNA sequencing. DNA was extracted using a blood cell DNA extraction kit (Saibaisheng, China) after 1 h of digestion at 30 °C by 4 mg/ml lysozyme (Tiangen, China) in 500-μl 50-mM EDTA buffer (pH 8.0). 16S ribosomal RNA (rRNA) genes were amplified with primers 8F (5′-GCGGATCCGC GGCCGCTGCAGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′-GGCTCGAGCGGCCGCCCGGGTTACCTTG TTACGACTT-3′) [65]. PCR reactions were performed on an Eppendorf Mastercycler Gradient (Eppendorf, Germany). The reaction mixture contained 1.2 μl of dNTPs (10 mM each), 5 μl of 10× PCR buffer (with $MgCl_2$), 2 μl of each primer (10 μM each), 0.8 μl Taq polymerase (5 U/μl) (TaKaRa, China), and 10–100 ng of DNA adjusted to 50 μl with sterilized deionized water. The reaction conditions were 94 °C for 5 min; 35 cycles of 30 s at 94 °C, 30 s at 51 °C, and 1 min 30 s at 72 °C; and a final extension at 72 °C for 10 min. PCR products with expected size of 1500 bp were visualized on 1 % agarose gels and purified by Axygen DNA Gel Extraction Kit (Axygen, USA) and sequenced in two directions on an ABI 3730XL DNA Analyzer (Applied Biosystems, USA) using the same primers. Consensus sequences were manually assembled and edited according to chromatograms in MEGA5 [58]. Alignments were done online using the EzTaxon-e server (http://www.eztaxon-e.ezcloud.net/) [29] and BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences in this study were deposited in the GenBank database (Table 3).

The 16S rRNA sequences of the 57 bacterial isolates were phylogenetically analyzed. Additional 70 sequences from the two databases mentioned above, most of which were that of type strains and ecologically related strains, were added and aligned using Clustal X [60], followed by manual refinement in BIOEDIT [21]. jModeltest 2.1 [12] showed that the GTR+I+G model was the most appropriate model according to the Akaike information criterion [42]. The phylogeny was constructed by the maximum likelihood approach using RAXML version 7.4.2 [53]. Confidence at each node was assessed by 1000 bootstrap replicates [22]. *Anabaena affinis* (AF247591) was used as outgroup. The resulting tree was visualized and edited with TreeGraph 2 [54] and refined with Adobe Illustrator CS3.

Sixteen beetle gut bacterial isolates representing 16 species were chosen to conduct the following experiments. If there were more than one morphotype in one species, one isolate in the most frequently isolated morphotype was randomly chosen. They were *Bacillus aryabhattai* (B34, KJ781859), *Bacillus* sp. (B42, KJ781865), *Bacillus safensis* (B31, KJ781872), *Delftia* sp. (B201, KJ781877), *Enterococcus faecalis* (B324, KJ781881), *Erwinia* sp. 1 (B44, KJ781883), *Erwinia* sp. 2 (B209, KJ781886), *Herbaspirillum chlorophenolicum* (B210, KJ781892), *Lactococcus lactis* (B39, KJ781894), *Pseudomonas* sp. 1 (B204, KJ781907), *Pseudomonas* sp. 5 (B27, KJ781912), *Pseudomonas* sp. 6 (B316, KJ781914), *Pseudomonas* sp. 11 (B330, KJ781935), *Rahnella aquatilis* (B35, KJ781939), *Rhodococcus* sp. (B211, KJ781947), and *Serratia* sp. (B326, KJ781959).

Conversion Experiments

We calculated the *cis*-verbenol concentration in a beetle's hindgut using the ratio of *cis*-verbenol in the hindgut (10^0 to 10^3 ng) [8, 70] and the estimated hindgut volume (1.21± 0.48 μl, n=14 (7♀, 7♂)). Gut volumes were estimated by measuring outer diameter of the respective gut sections [31]. The *cis*-verbenol concentration in the hindgut was estimated to occur in a range from 10^0 to 10^3 ng/μl. Using this information, we set 4, 40, and 200 ng/μl as *cis*-verbenol concentrations in the conversion experiments.

The 16 bacterial isolates were grown in M9 minimal medium. A dilution of 1:100 of each isolate was made when cultures were adjusted to an optical density $(OD)_{600}$ of 0.5. After 12-h incubation, *cis*-verbenol dissolved in dimethyl sulfoxide (DMSO) was added to a 4-ml bacterial suspension (final concentration 4, 40, and 200 ng/μl) and shaken for further 24 h. A suspension containing equivalent *cis*-verbenol without bacteria was run as a control in the same manner for each group. All solutions (n=5–8) were extracted with hexane and then stored for the chemical analysis.

Table 1 Volatile chemical production in *Dendroctonus valens* guts and frass in control and antibiotic-treated media amended with α-pinene after 96 h of feeding

	cis-Verbenol		*trans*-Verbenol		Myrtenol		Verbenone	
	Control gut/frass	Antibiotic gut/frass	Control gut/frass	Antibiotic gut/frass	Control gut/frass	Antibiotic gut/frass	Control gut/frass	Antibiotic gut/frass
Male 1	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 2	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 3	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 4	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 5	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 6	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 7	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 8	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 9	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 10	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Male 11	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 1	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 2	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 3	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 4	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 5	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 6	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 7	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 8	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 9	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 10	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 11	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Female 12	+/−	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Summary	23(+)/23(−)	23(+)/23(+)	23(+)/23(+)	23(+)/23(+)	23(+)/23(+)	23(+)/23(+)	23(+)/23(+)	23(+)/23(+)

"+" or "−" designates the presence or absence of a specific volatile

Antibacterial Assessment of Chemicals

The minimum inhibitory concentration (MIC) of the chemicals (*cis*-verbenol and verbenone) against the isolates was used to assess whether bacteria benefit from the conversion. MIC of the chemicals was determined using a broth microdilution method modified by Cosentino et al. [11]. All tests were performed in TSB, and serial dilutions of each chemical dissolved in DMSO were performed in a 96-well microtiter plate (Greiner, Germany) over the range of 225, 450, 675, 900, 1350, 1800, and

Table 2 Total amounts of chemicals quantified from adult *Dendroctonus valens* guts after 96-h antibiotic treatment versus control

	Volatiles	Control	Antibiotic	*P* values
Female (ng/beetle), *N*=12	*cis*-Verbenol	178.09±63.94	227.45±70.54	0.609[a]
	Verbenone	71.25±15.16	18.20±5.76	<0.001[b]
	trans-Verbenol	7.63±1.99	8.47±1.86	0.761[a]
	Myrtenol	25.72±9.46	12.86±3.30	0.525[b]
Male (ng/beetle), *N*=11	*cis*-Verbenol	15.02±3.31	8.51±0.78	0.061[b]
	Verbenone	30.89±6.45	14.48±2.37	0.002[b]
	trans-Verbenol	4.72±0.78	10.56±4.48	0.214[a]
	Myrtenol	10.50±2.55	8.63±3.45	0.667[a]

[a] Independent-samples *t* test

[b] Mann–Whitney *U* test was used

Table 3 GenBank accession numbers of isolates from adult *Dendroctonus valens* guts and frass in China in this study and similarity scores to closest type strains and ecologically related strains in NCBI according to the 16S rDNA

Isolate numbers	Accession numbers	Closest type strains and ecologically related strains[a]	Species affiliation	Isolate source	Similarity (%)
			Actinobacteria		
			Sterptomycetaceae		
			Cellulomonadaceae		
B419	KJ781874	*Cellulomonas hominis* X82598 T	*Cellulomonas* sp.	Frass	99.1
			Microbacteriaceae		
B136	KJ781896	*Leucobacter alluvii* AM072820 T	*Le. alluvii*	Frass	99.4
B120, B138	KJ781897–KJ781898	*Microbacterium foliorum* AJ249780 T	*Microbacterium* sp.	Frass	99.4
			Micrococcaceae		
B119	KJ781857	*Arthrobacter protophormiae* X80745 T	*Ar. protophormiae*	Frass	100
B60	KJ781899	*Micrococcus terreus* strain FJ423763 T	*M. terreus*	Frass	99.9
			Nocardiaceae		
B126, B211	KJ781946–KJ781947	*Rhodococcus qingshengii* DQ090961 T	*Rhodococcus* sp.	Gut/Frass	100
			Sterptomycetaceae		
B422	KJ781972	*Streptomyces aureus* AB249976 T	*Streptomyces* sp. 1	Frass	98.7
B410, B413	KJ781968–KJ781969	*Streptomyces rishiriensis* AB184383 T	*Str. rishiriensis*	Frass	99.4
B425	KJ781976	*Streptomyces thinghirensis* FM202482 T	*Streptomyces* sp. 3	Frass	98.9
B424	KJ781978	*Streptomyces candidus* DQ026663 T	*Streptomyces* sp. 4	Frass	98.7
B414	KJ781982	*Streptomyces cocklensis* FR692107 T	*Streptomyces* sp. 8	Frass	98.9
B411	KJ781986	*Streptomyces subrutilus* X80825T	*Streptomyces* sp. 12	Frass	99.7
			Firmicutes		
			Bacillaceae		
B23, B34, B43	KJ781858–KJ781860	*Bacillus aryabhattai* EF114313 T	*B. aryabhattai*	Gut/Frass	100
B41	KJ781871	*Bacillus nealsonii* EU656111 T	*B. nealsonii*	Frass	99.4
B31, B32	KJ781872–KJ781873	*Bacillus safensis* AF234854 T	*B. safensis*	Gut	100
B8, B36, B37, B40, B42, B47, B48, B92, B107	KJ781861- KJ781869	*Bacillus cereus* AE016877 T	*Bacillus* sp.	Gut/Frass	99.7–99.9
B111, B217	KJ781889- KJ781890	*Exiguobacterium undae* DQ019165 T	*Ex. undae*	Frass	99.9
			Enterococcaceae		
B324	KJ781881	*Enterococcus faecalis* AB012212 T	*En. faecalis*	Gut	100
			Paenibacillaceae		
B62, B135	KJ781901, KJ781903	*Paenibacillus xylanexedens* EU558281 T	*Paenibacillus* sp.	Frass	99.2
B94	KJ781900	*Paenibacillus odorifer* AJ223990 T	*Pae. odorifer*	Frass	98.5
			Streptococcaceae		
B38, B39	KJ781893- KJ781894	*Lactococcus lactis* AB100803 T	*La. lactis*	Gut/Frass	100
			β-Proteobacteria		
			Comamonadaceae		
B201, B203, B208	KJ781877- KJ781879	*Delftia acidovorans* AF078774 T	*Delftia* sp.	Gut/Frass	99.4–99.5
			Oxalobacteraceae		
B210	KJ781892	*Herbaspirillum chlorophenolicum* AB094401 T	*H. chlorophenolicum*	Gut	99.9
			γ-Proteobacteria		
			Enterobacteriaceae		
B98	KJ781880	*Raoultella terrigena* Y17658 T	Enterobacteriaceae bacterium	Frass	99.4
B15, B44	KJ781882–KJ781883	*Erwinia* sp. FJ811868 (*Dendroctonus. valens*)	*Erwinia* sp. 1	Gut/Frass	99.7
		Erwinia psidii Z96085 T			96.8

Table 3 (continued)

Isolate numbers	Accession numbers	Closest type strains and ecologically related strains[a]	Species affiliation	Isolate source	Similarity (%)
B209	KJ781886	*Pantoea* sp. FJ811870 (*D. valens*)	*Erwinia* sp. 2	Gut/Frass	99.3
		Erwinia toletana FR870447 T			97.1
B137	KJ781905	*Pantoea agglomerans* AJ233423 T	*Pan. agglomerans*	Frass	99.8
B35, B215, B216	KJ781939–KJ781941	*Rahnella aquatilis* FJ811859 (*D. valens*)	*Rah. aquatilis*	Gut/Frass	98.3–99.7
		Rah. aquatilis CP003244 T			97.2–97.9
B131	KJ781962	*Serratia plymuthica* AJ233433 T	*Se. plymuthica*	Frass	99.8
B99	KJ781963	*Serratia proteamaculans* FJ811861 (*D. valens*)	*Se. proteamaculans*	Frass	99.6
		Serratia proteamaculans AJ233434 T			99.9
B7, B21, B97, B115, B213, B214, B326, B328, B329	KJ781948–KJ781953	*Se. liquefaciens* FJ811864 (*D. valens*)	*Serratia* sp.	Gut/Frass	99.7
	KJ781959–KJ781961	*Se. liquefaciens* CP 006252 T			99.7
			Moraxellaceae		
B108	KJ781855	*Acinetobacter guillouiae* X81659 T	*Ac. guillouiae*	Frass	99.8
			Pseudomonadaceae		
B204	KJ781907	*Pseudomonas nitroreducens* AM088474 T	*Pseudomonas* sp. 1	Gut	99.6
B85, B86	KJ781909–KJ781910	*Pseudomonas koreensis* AF468452 T	*Pseudomonas* sp. 3	Frass	99.9
B27	KJ781912	*Pseudomonas mandeliii* AF058286 T	*Pseudomonas* sp. 5	Gut/Frass	99.6
B219, B316	KJ781913–KJ781914	*Pseudomonas thivervalensis* AF100323 T	*Pseudomonas* sp. 6	Gut/Frass	99.1, 99.3
B145	KJ781915	*Pseudomonas extremaustralis* AHIP01000073 T	*Pseudomonas* sp. 7	Frass	99.8
B116, B117, B122	KJ781916–KJ781918	*Pseudomonas azotoformans* D84009 T	*Pseudomonas* sp. 8	Frass	99.7
B82, B129	KJ781919–KJ781920	*Pseudomonas ficuserectae* AB021378 T	*Pseudomonas* sp. 9	Frass	99.5
B331	KJ781921	*Pseudomonas palleroniana* AY091527T	*Pseudomonas* sp. 10	Frass	99.5
B90, B93, B109, B113, B140, B142, B144, B212, B218, B325, B327, B330, B332, B416, B420	KJ781922–KJ781930 KJ781933–KJ781938	*Pseudomonas brenneri* AF268968 T	*Pseudomonas* sp. 11	Gut/Frass	99.6–99.7
			Xanthomonadaceae		
B96, B110	KJ781964–KJ781965	*Stenotrophomonas maltophilia* FJ811851	*Ste. maltophilia*	Frass	98.0–99.0
		Ste. maltophilia AB008509 T			

"T" indicates type strain

2700 ng/μl. Overnight broth cultures were prepared in TSB. A dilution 1:100 of bacterial suspension ($OD_{600}\approx0.5$) was added to each well. The plates were incubated aerobically at 25 °C for 12 h and then MIC was determined. Each treatment for each compound at each concentration, including the control, was replicated for three times.

Chemical Analysis

Extracts (2 μl) were injected splitless into a GC-MS (Agilent 6980N GC coupled 5973 mass selective detector) equipped with an HP5-MS capillary column (0.25-mm i.d.×60 m; Agilent Technologies, Inc., Palo Alto, CA, USA), and the column temperature was programmed from an initial temperature of 50 °C for 1 min and then increased by 5 °C/min to 100 °C, by 3 °C/min to 130 °C, and by 20 to 320 °C and held for 2 min. Components of the extracts were identified by comparing retention times and mass spectra with authentic standards and those in the NIST02 library (Scientific Instrument Services, Inc., Ringoes, NJ, USA). Quantification was performed using an internal standard (heptyl acetate) that was added to each sample.

Statistical Analysis

Prior to analysis, we tested all variables for normality with the Kolmogorov–Smirnov test and homogeneity of group

variances with Levene's test. In comparisons of gut volatiles, means of two groups of cases were tested using the independent-samples *t* test or Mann–Whitney *U* test, depending on the results of the test of normality and homogeneity of variance. In conversion experiments, we initially used the Scheirer–Ray–Hare extension of the Kruskal–Wallis test as variances were unequal even after data transformations [16]; then, Dunnett's T3 test was used for post hoc comparisons. Using the MIC values, the descriptive statistics (median, MIC_{50}, MIC_{90}, mode, range, and susceptibility) of *cis*-verbenol and verbenone were calculated for each isolate [30]. The MICs of *cis*-verbenol and verbenone were compared using Mann–Whitney *U* test [30, 67]. All data were analyzed using SPSS 12.0 (SPSS, Inc., Chicago, IL, USA) for Windows.

Results

Volatiles in *D. valens* Guts and Frass from Control and Antibiotic Treatment

In the control treatment, myrtenol, *trans*-verbenol, and verbenone were detected in 100 % of tested *D. valens* guts and frass in phloem media. On the other hand, while *cis*-verbenol was detected in 100 % of the guts of both control and treated beetles and in all of the frass samples from treated beetles, none of the control beetles had detectable *cis*-verbenol in their frass (Table 1). In the antibiotic treatment, all these volatiles could be detected in 100 % of tested *D. valens* guts and frass (Table 1). Quantification analyses showed that gut verbenone amounts were significantly reduced after antibiotic treatment (male, from 30.89±6.45 to 14.48±2.37 ng/beetle; female, from 71.25±15.16 to 18.20±5.76 ng/beetle), while no significant changes existed in *cis*-verbenol, *trans*-verbenol, and myrtenol amounts (Table 2). No microbial CFU in TSA were detected in samples of guts and frass in the antibiotic treatment, while we found $1.03\pm0.30\times10^7$ of microbial CFU/gut and $1.35\pm0.31\times10^7$ of CFU/frass in the control group.

Isolation and Identification of Bacterial Species

A total of 501 bacterial isolates from 119 gut samples and 673 isolates from 40 frass samples were selected and purified. BLAST results and phylogenetic analyses (data not shown) identified 42 species, belonging to 22 genera in 15 families in three phyla (Table 3). The three phyla were *Proteobacteria* (21 species) including γ-*Proteobacteria* (19 species) and β-*Proteobacteria* (two species), *Firmicutes* (nine species), and *Actinobacteria* (12 species) (Table 3). Sixteen of these species were isolated in *D. valens* guts, and their phylogeny was shown (Table 3 and Fig. S1). Among them, 12 bacterial species were isolated from both *D. valens* guts and frass, accounting for 98.0 and 87.5 % of the total isolates, respectively (Fig. 1). The most frequently isolated species both in guts and frass were *Pseudomonas* sp. 11 (gut, 29.7 %; frass, 32.2 %), *Serratia* sp. (gut, 29.5 %; frass, 17.4 %), and *Rah. aquatilis* (gut, 25.8 %; frass, 19.9 %). The less frequently isolated species were *Erwinia* sp. 2 (gut, 5.6 %; frass, 5.1 %), *Erwinia* sp. 1 (gut, 0.6 %; frass, 3.9 %), *Bacillus* sp. (gut, 3.6 %; frass, 1.6 %), *B. aryabhattai* (gut, 0.6 %; frass, 1.6 %), *Delftia* sp. (gut, 1.2 %; frass, 0.2 %), and *Pseudomonas* sp. 6 (gut, 0.2 %; frass, 4.6 %) with the remaining species each accounting for less than 1 % (Fig. 1). *H. chlorophenolicum* (gut, 0.2 %), *En. faecalis* (gut, 0.6 %), *Pseudomonas* sp. 1 (gut, 0.4 %), and *B. safensis* (gut, 0.8 %) were isolated only in *D. valens* gut.

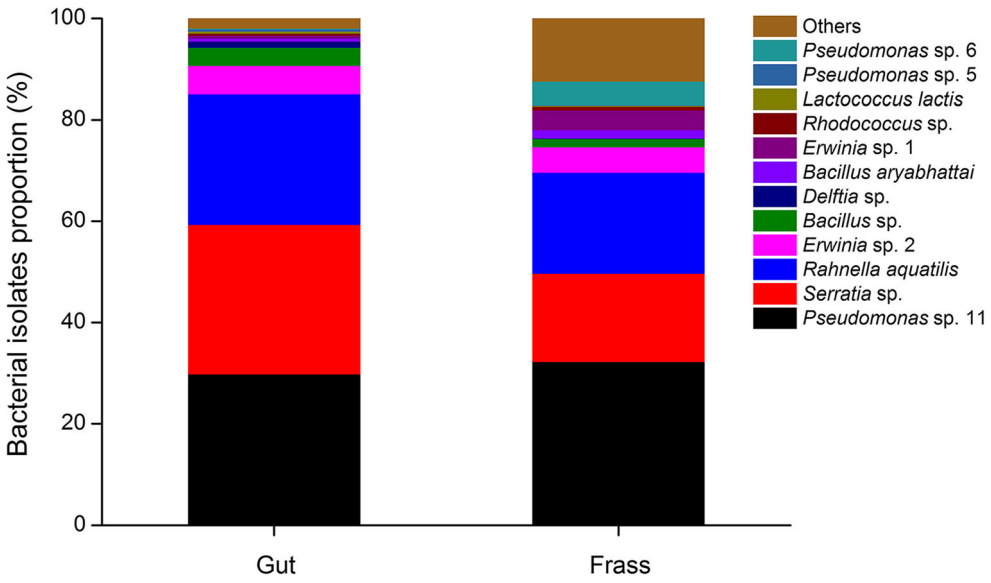

Fig. 1 The composition and relative proportion of bacterial isolates from Chinese *Dendroctonus valens* guts and frass. Species with fewer than 1 % were pooled as "others"

Table 4 Results of the Scheirer-Ray-Hare test with verbenone production as dependent variable and bacterial isolates and *cis*-verbenol concentrations as independent variables

Source	DF	SS	H	*P* value
cis-Verbenol concentrations	2	340,174.14	57.81	<0.001
Bacterial isolates	16	1,111,817.37	188.93	0.000
Bacterial isolates × *cis*-verbenol concentrations	32	126,356.57	21.47	0.921

DF degrees of freedom, *SS* sum of squares, *H* Scheirer-Ray-Hare non-parametric two-way analysis of variance statistic

Conversion Experiments

The results of the Scheirer–Ray–Hare test revealed a significant effect of bacterial isolates and *cis*-verbenol concentrations on verbenone production, while no interaction was found between the two factors (Table 4). Incubation of *D. valens* bacterial isolates with 4, 40, and 200 ng/μl of *cis*-verbenol resulted in respective yields of verbenone shown in Fig. 2. Thirteen out of the 16 bacterial isolates significantly converted *cis*-verbenol to verbenone at three concentrations than control (Fig. 2). No verbenone was detected in control treatments. Incubation of B39 (*La. lactis*) with three concentrations of *cis*-verbenol yielded 3.49±0.08, 26.16±0.46, and 114.05±3.37 ng/μl of verbenone, which was the highest level of verbenone production among 16 tested species. B330 (*Pseudomonas* sp. 11), B326 (*Serratia* sp.), and B35 (*Rah. aquatilis*), representing the three most frequently isolated species in guts and frass, also had *cis*-verbenol conversion ability. No verbenone was produced by incubation of B44 (*Erwinia* sp. 1), B209 (*Erwinia* sp. 2), and B31 (*B. safensis*) with *cis*-verbenol. The production of verbenone by B330 (*Pseudomonas* sp. 11) was significantly higher than that by B27 (*Pseudomonas* sp. 5) and B316 (*Pseudomonas* sp. 6) at three concentrations (Fig. 2). The verbenone production by

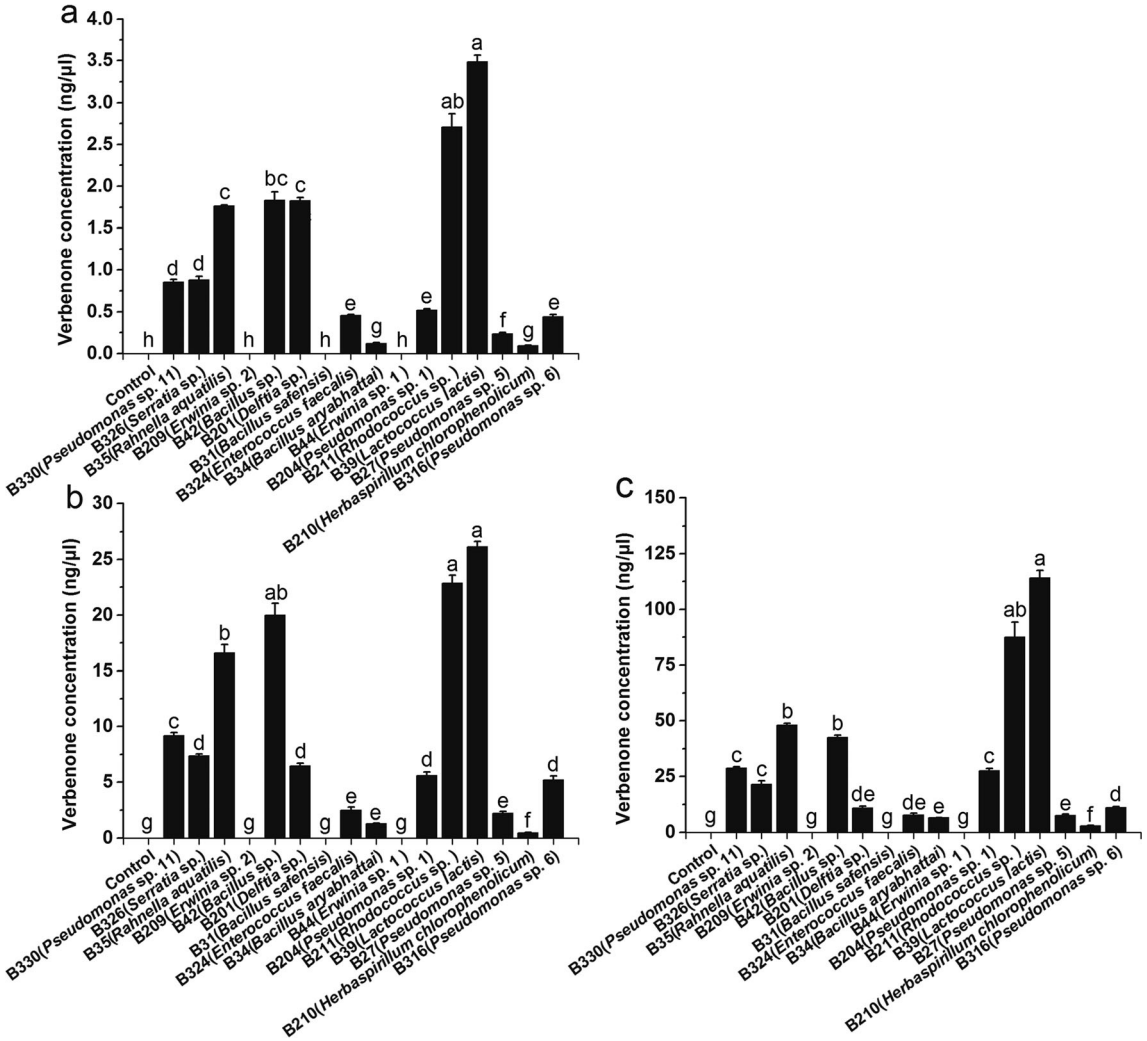

Fig. 2 The amounts of verbenone produced by Chinese *Dendroctonus valens* gut bacterial isolates at three *cis*-verbenol concentrations. The bacterial isolates were arranged according to the frequency of isolation in *D. valens* gut. *Labels with different letters* are significantly different at *P*=0.05, Dunnett's T3 test

Table 5 Minimum inhibitory concentrations (ng/μl) of *cis*-verbenol and verbenone against the 16 bacterial isolates

Isolate numbers	Species	*cis*-Verbenol	Verbenone
B211	*Rhodococcus* sp.	900	2700
B34	*Bacillus aryabhattai*	900	2700
B31	*Bacillus safensis*	900	>2700
B42	*Bacillus* sp.	900	1800
B324	*Enterococcus faecalis*	1350	>2700
B39	*Lactococcus lactis*	900	2700
B201	*Delftia* sp.	225	675
B210	*Herbaspirillum chlorophenolicum*	225	675
B44	*Erwinia* sp. 1	1350	2700
B209	*Erwinia* sp. 2	675	1800
B35	*Rahnella aquatilis*	900	1800
B326	*Serratia* sp.	1350	2700
B204	*Pseudomonas* sp. 1	1350	2700
B27	*Pseudomonas* sp. 5	675	1800
B316	*Pseudomonas* sp. 6	900	2700
B330	*Pseudomonas* sp. 11	900	2700

three *Bacillus* isolates (B31, B34, and B42) varied significantly at three concentrations (Fig. 2).

Antibacterial Assessments of Chemicals

All the 16 bacterial isolates were subjected to antibacterial assessments. There was no visible effect on the growth of tested bacteria in suspension containing DMSO compared with control (DMSO-free suspension), suggesting that the addition of DMSO did not affect the growth of any tested bacterial isolates. The results of MIC tests showed that *cis*-verbenol exhibited stronger antimicrobial activities than verbenone for all 16 isolates ($U=-3.92$, $P<0.001$). The MIC range of *cis*-verbenol to the tested bacteria was from 225 to 1350 ng/μl, while the range of verbenone was from 675 to >2700 ng/μl (Tables 5 and 6). The medians, MIC_{50}s, MIC_{90}s, and modes for *cis*-verbenol were lower than those for verbenone (Table 5). B210 (*H. chlorophenolicum*) and B201 (*Delftia* sp.) were the most sensitive isolates to *cis*-verbenol and verbenone among 16 tested isolates. B324 (*En. faecalis*), B44 (*Erwinia* sp. 1), B326 (*Serratia* sp.), and B204 (*Pseudomonas* sp. 1) were the most tolerant to *cis*-verbenol among 16 tested isolates. B324 (*En. faecalis*) and B31 (*B. safensis*) were the most tolerant to verbenone among 16 tested isolates.

Discussion

This study shows for the first time that gut-associated bacteria of *D. valens* are capable of verbenone production *in vitro* at three concentrations of the verbenone precursor *cis*-verbenol (Fig. 2). Considering that attack behavior of *D. valens* is partially mediated by volatiles emanating from the bark beetle's gut in combination with host tree monoterpenes [17, 35, 56, 57, 72], and one of the volatiles is verbenone, which serves as an attractant at low levels but as a repellent at high concentrations [72], our findings provide a clue that verbenone production by associated bacteria of *D. valens* may help beetles produce pheromone and further regulate beetle's attack behavior. The conversion experiments were conducted in vitro under aerobic conditions, since, to our knowledge, no direct evidence has been shown whether the bark beetle gut is aerobic or anaerobic. Future studies are needed to show what conditions occur in the gut and how that influences bacterial function.

In this study, examination of *D. valens* gut volatiles showed that antibiotic-treated beetles produced significantly lower amounts of verbenone than beetles in control group, while no significant difference was found for the other three gut volatiles (Table 2), which suggested that the microorganisms may be involved in gut verbenone production. In addition, at the three *cis*-verbenol concentrations (4, 40, and 200 ng/μl) (Fig. 2) which fall within an estimated beetle gut concentration range of *cis*-verbenol (calculated *cis*-verbenol concentration range 10^0–10^3 ng/μl), 13 out of 16 bacterial isolates were all capable of verbenone production in the bioconversion assay. These results suggest that gut-associated microbiota are indeed involved in verbenone production in *D. valens* guts in vivo, but more experiments are needed to reveal whether the verbenone produced by *D. valens*-associated bacteria regulates the bark beetle's behavior under field conditions.

The findings of this study also suggest that continual verbenone production may cause this compound to build up in frass. No *cis*-verbenol was detected in *D. valens* frass in the

Table 6 Descriptive statistics of minimum inhibitory concentrations (ng/μl) of *cis*-verbenol and verbenone against the 16 bacterial isolates

Antibacterial agent	No.	Median	MIC_{50}	MIC_{90}	Mode*	MIC range	Susceptibility
cis-Verbenol	16	900	900	1350	900	225–1350	100 %
Verbenone	16	2700	2700	2700	2700	675–>2700	87.5 %

MIC_{50} = antibiotic concentration that would inhibit the growth of 50 % of the tested bacterial isolates; MIC_{90} = antibiotic concentration that would inhibit the growth of 90 % of the tested bacterial isolates; median = the value in the middle of the rank; mode* = the value among all observations that occurs at the greatest frequency

control treatment, but it was found in the antibiotic treatment (Table 1), and 10 out of 12 bacterial species shared between *D. valens* guts and frass (Fig. 1) were capable of verbenone production (Fig. 2), both supporting this conclusion. Insect frass is a good nutrient source with various volatiles available for commensal microorganisms [14], providing good conditions for microorganisms to convert excreted *cis*-verbenol to verbenone pheromone. Commensal gut microbiota of locusts were previously shown to continually produce aggregation pheromones in frass [13–15]. *Klebsiella oxytoca* and several *Bacillus* species in the feces of *Acrolepiopsis assectella* produced kairomones that attract parasitoids [59]. Volatiles in *D. valens* frass have been illustrated to affect adult beetles' behavior [9, 34]. New studies should explore whether the volatiles, including verbenone, are produced by gut-associated bacteria in *D. valens* frass and mediate the beetle behavior.

The most frequent isolates with Chinese *D. valens* in this study were bacteria in the genera *Pseudomonas*, *Serratia*, *Rahnella*, *Erwinia*, *Bacillus*, and *Delftia* (Fig. 1), all of which are common bark beetle gut associates [40, 51, 62, 71]. Among them, *Rah. aquatilis* and bacteria in the genera *Serratia* and *Erwinia* have been isolated frequently in *D. valens* collected in Mexico [39], and *Rah. aquatilis* and bacteria in the genera *Bacillus* and *Delftia* were also detected in *D. valens* from America [1]. Many of them can metabolize monoterpenes of host pines [4, 6, 45], and the abilities of different microorganisms to reduce concentrations of different terpenes appear complementary to each other. For example, *Rahnella* reduced α-pinene by more than 40 %, and *Serratia* reduced by 55–75 % the concentrations of many monoterpenes applied to media with the exception of α-pinene [4, 45]. Here, in pilot experiments, no pheromones were detected when culturing all 16 gut bacterial species isolated from *D. valens* with α-pinene, β-pinene, D-limonene, myrcene, or 3-carene, though some of them could reduce concentrations of these terpenes compared to control (data not shown). While the verbenol presumably produced by bark beetles [7, 8, 24, 25] was further metabolized to the pheromone verbenone by 13 out of 16 *D. valens* gut bacterial isolates, species with close phylogenetic relationships did not show similar conversion capabilities.

Generally, the cytotoxic activity of alcohols is much higher than that of related ketones [2], so the accumulation of the terpene alcohol verbenol could be more harmful to gut bacteria than the corresponding ketone, verbenone. This trend was confirmed through the antibacterial assessments of chemicals with tested *cis*-verbenol having a concentration similar to that in their surroundings (Table 5), suggesting that beetle gut bacteria have the ability to adapt to adverse environmental conditions [4, 28, 48]. Thus, we hypothesize that this conversion is beneficial to gut bacteria themselves by reducing cytotoxicity from the precursor *cis*-verbenol to the verbenone pheromone, while it also benefits *D. valens* by yielding its multifunctional pheromone verbenone. These hypotheses should be further tested in this and other insect-gut bacteria systems.

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References

1. Adams AS, Adams SM, Currie CR, Gillette NE, Raffa KF (2010) Geographic variation in bacterial communities associated with the red turpentine beetle (Coleoptera: Curculionidae). Environ Entomol 39:406–414
2. Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils-a review. Food Chem Toxicol 46:446–475
3. Blomquist GJ, Figueroa-Teran R, Aw M, Song M, Gorzalski A, Abbott NL, Chang E, Tittiger C (2010) Pheromone production in bark beetles. Insect Biochem Mol Biol 40:699–712
4. Boone CK, Keefover-Ring K, Mapes AC, Adams AS, Bohlmann J, Raffa KF (2013) Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. J Chem Ecol 39: 1003–1006
5. Brand J, Bracke J, Britton L, Markovetz A, Barras S (1976) Bark beetle pheromones: production of verbenone by a mycangial fungus of *Dendroctonus frontalis*. J Chem Ecol 2:195–199
6. Brand JM, Bracke JM, Markovetz AJ, Wood DL, Browne LE (1975) Production of verbenol pheromone by a bacterium isolated from bark beetles. Nature 254:136–137
7. Bridges JR (1982) Effects of juvenile hormone on pheromone synthesis in *Dendroctonus frontalis*. Environ Entomol 11:417–420
8. Byers JA, Wood DL (1981) Antibiotic-induced inhibition of pheromone synthesis in a bark beetle. Science 213:763–764
9. Chen HF, Salcedo C, Sun JH (2012) Male mate choice by chemical cues leads to higher reproductive success in a bark beetle. Anim Behav 83:421–427
10. Conn JE, Borden JH, Hunt DWA, Holman J, Whitney HS, Spanier OJ, Pierce HD Jr, Oehlschlager AC (1984) Pheromone production by axenically reared *Dendroctonus ponderosae* and *Ips paraconfusus* (Coleoptera: Scolytidae). J Chem Ecol 10:281–290
11. Cosentino S, Tuberoso C, Pisano B, Satta M, Mascia V, Arzedi E, Palmas F (1999) In-vitro antimicrobial activity and chemical composition of Sardinian *thymus* essential oils. Lett Appl Microbiol 29: 130–135
12. Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nat Meth 9: 772
13. Dillon R, Vennard C, Charnley A (2002) A note: gut bacteria produce components of a locust cohesion pheromone. J Appl Microbiol 92:759–763
14. Dillon RJ, Dillon VM (2004) The gut bacteria of insects: nonpathogenic interactions. Annu Rev Entomol 49:71–92

15. Dillon RJ, Vennard CT, Charnley AK (2000) Pheromones: exploitation of gut bacteria in the locust. Nature 403:851
16. Dytham C (2011) Choosing and using statistics: a biologist's guide. Wiley-Blackwell, Oxford
17. Erbilgin N, Mori SR, Sun JH, Stein JD, Owen DR, Merrill LD, Bolanos RC, Raffa KF, Montiel TM, Wood DL, Gillette NE (2007) Response to host volatiles by native and introduced populations of *Dendroctonus valens* (Coleoptera: Curculionidae, Scolytinae) in North America and China. J Chem Ecol 33:131–146
18. Fettig CJ, Borys RR, Dabney CP, McKelvey SR, Cluck DR, Smith SL (2005) Disruption of red turpentine beetle attraction to baited traps by the addition of California fivespined *Ips* pheromone components. Can Entomol 137:748–752
19. Gillette NE, Stein JD, Owen DR, Webster JN, Fiddler GO, Mori SR, Wood DL (2006) Verbenone-releasing flakes protect individual Pinus contorta trees from attack by *Dendroctonus ponderosae* and *Dendroctonus valens* (Coleoptera: Curculionidae, Scolytinae). Agric For Entomol 8:243–251
20. Gitau CW, Bashford R, Carnegie AJ, Gurr GM (2013) A review of semiochemicals associated with bark beetle (Coleoptera: Curculionidae: Scolytinae) pests of coniferous trees: a focus on beetle interactions with other pests and their associates. For Ecol Manage 297:1–14
21. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98
22. Hillis DM, Bull JJ (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol 42:182–192
23. Hughes PR (1973) *Dendroctonus*: production of pheromones and related compounds in response to host monoterpenes. Z Angew Entomol 73:294–312
24. Hughes PR (1973) Effect of α-pinene exposure on *trans*-verbenol synthesis in *Dendroctonus ponderosae* Hopk. Naturwissenschaften 60:261–262
25. Hunt D, Borden J (1989) Terpene alcohol pheromone production by *Dendroctonus ponderosae* and *Ips paraconfusus* (Coleoptera: Scolytidae) in the absence of readily culturable microorganisms. J Chem Ecol 15:1433–1463
26. Hunt DWA, Borden JH (1990) Conversion of verbenols to verbenone by yeasts isolated from *Dendroctonus ponderosae* (Coleoptera: Scolytidae). J Chem Ecol 16:1385–1397
27. Jurenka R (2004) Insect pheromone biosynthesis. Top Curr Chem 239:97–132
28. Kaufman MG, Klug MJ (1991) The contribution of hindgut bacteria to dietary carbohydrate utilization by crickets (Orthoptera: Gryllidae). Comp Biochem Physiol A Physiol 98:117–123
29. Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62: 716–721
30. Kowalski RP, Yates KA, Romanowski EG, Karenchak LM, Mah FS, Gordon YJ (2005) An ophthalmologist's guide to understanding antibiotic susceptibility and minimum inhibitory concentration data. Ophthalmology 112:1987.e1–1987.e5
31. Lemke T, Stingl U, Egert M, Friedrich MW, Brune A (2003) Physicochemical conditions and microbial activities in the highly alkaline gut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl Environ Microbiol 69:6650–6658
32. Leufvén A, Bergström G, Falsen E (1984) Interconversion of verbenols and verbenone by identified yeasts isolated from the spruce bark beetle *Ips typographus*. J Chem Ecol 10:1349–1361
33. Levinson HZ (1975) Possibilities of using insectistatics and pheromones in pest control. Naturwissenschaften 62:272–282
34. Liu Z, Zhang L, Sun J (2006) Attacking behavior and behavioral responses to dust volatiles from holes bored by the red turpentine beetle, *Dendroctonus valens* (Coleoptera: Scolytidae). Environ Entomol 35:1030–1036
35. Liu Z, Wang B, Xu B, Sun J (2011) Monoterpene variation mediated attack preference evolution of the bark beetle *Dendroctonus valens*. PLoS ONE 6:e22005
36. Lou QZ, Lu M, Sun JH (2014) Yeast diversity associated with invasive *Dendroctonus valens* killing *Pinus tabuliformis* in China using culturing and molecular methods. Microb Ecol 68:397–415
37. Matsuura K (2001) Nestmate recognition mediated by intestinal bacteria in a termite, *Reticulitermes speratus*. Oikos 92:20–26
38. Miller DR, Lafontaine JP (1991) *cis*-Verbenol: an aggregation pheromone for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). J Entomol Soc Brit Columbia 88:34–38
39. Morales-Jimenez J, Zuniga G, Villa-Tanaca L, Hernandez-Rodriguez C (2009) Bacterial community and nitrogen fixation in the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae). Microb Ecol 58:879–891
40. Morales-Jimenez J, Zuniga G, Ramirez-Saad HC, Hernandez-Rodriguez C (2012) Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright (Curculionidae: Scolytinae) and their cellulolytic activities. Microb Ecol 64:268–278
41. Paine TD, Raffa KF, Harrington TC (1997) Interactions among scolytid bark beetles, their associated fungi, and live host conifers. Annu Rev Entomol 42:179–206
42. Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst Biol 53: 793–808
43. Pureswaran DS, Sullivan BT, Ayres MP (2006) Fitness consequences of pheromone production and host selection strategies in a tree-killing bark beetle (Coleoptera: Curculionidae: Scolytinae). Oecologia 148:720–728
44. Qiu J (2013) China battles army of invaders. Nature 503:450–451
45. Raffa KF (2014) Terpenes tell different tales at different scales: glimpses into the chemical ecology of conifer-bark beetle-microbial interactions. J Chem Ecol 40:1–20
46. Raffa KF, Berryman AA (1987) Interacting selective pressures in conifer-bark beetle systems: a basis for reciprocal adaptations? Am Nat 129:234–262
47. Rappaport NG, Owen DR, Stein JD (2001) Interruption of semiochemical-mediated attraction of *Dendroctonus valens* (Coleoptera: Scolytidae) and selected nontarget insects by verbenone. Environ Entomol 30:837–841
48. Santo Domingo J, Kaufman M, Klug M, Holben W, Harris D, Tiedje J (1998) Influence of diet on the structure and function of the bacterial hindgut community of crickets. Mol Ecol 7:761–767
49. Savithiry N, Gage D, Fu WJ, Oriel P (1998) Degradation of pinene by *Bacillus pallidus* BR425. Biodegradation 9:337–341
50. Schlyter F, Zhang QH, Liu GT, Ji LZ (2001) A successful case of pheromone mass trapping of the bark beetle *Ips duplicatus* in a forest island, analysed by 20-year time-series data. Integr Pest Manag Rev 6:185–196
51. Six D (2013) The bark beetle holobiont: why microbes matter. J Chem Ecol 39:989–1002
52. Spiteller D, Dettner K, Boland W (2000) Gut bacteria may be involved in interactions between plants, herbivores and their predators: microbial biosynthesis of N-acylglutamine surfactants as elicitors of plant volatiles. Biol Chem 381:755–762
53. Stamatakis A, Ludwig T, Meier H (2005) RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics 21:456–463

54. Stover B, Muller K (2010) TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. BMC Bioinform 11: 7
55. Sun J, Lu M, Gillette NE, Wingfield MJ (2013) Red turpentine beetle: innocuous native becomes invasive tree killer in China. Annu Rev Entomol 58:293–311
56. Sun J, Miao Z, Zhang Z, Zhang Z, Gillette NE (2004) Red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Scolytidae), response to host semiochemicals in China. Environ Entomol 33: 206–212
57. Sun J, Gillette NE, Miao Z, Kang L, Zhang Z, Owen DR, Stein JD (2003) Verbenone interrupts attraction to host volatiles and reduces attack on P*inus tabuliformis* (Pinaceae) by *Dendroctonus valens* (Coleoptera: Scolytidae) in the People's Republic of China. Can Entomol 135:721–732
58. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739
59. Thibout E, Guillot JF, Ferary S, Limouzin P, Auger J (1995) Origin and identification of bacteria which produce kairomones in the frass of *Acrolepiopsis assectella* (Lep., Hyponomeutoidea). Experientia 51:1073–1075
60. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
61. Touré AM, Mackey AJ, Wang ZX, Beier JC (2000) Bactericidal effects of sugar-fed antibiotics on resident midgut bacteria of newly emerged anopheline mosquitoes (Diptera: Culicidae). J Med Entomol 37:246–249
62. Vasanthakumar A, Delalibera I, Handelsman J, Klepzig KD, Schloss PD, Raffa KF (2006) Characterization of gut-associated bacteria in larvae and adults of the southern pine beetle, *Dendroctonus frontalis* Zimmermann. Environ Entomol 35:1710–1717
63. Vité JP, Francke W (1976) The aggregation pheromones of bark beetles: progress and problems. Naturwissenschaften 63:550–555
64. Wang B, Lu M, Cheng C, Salcedo C, Sun J (2013) Saccharide-mediated antagonistic effects of bark beetle fungal associates on larvae. Biol Lett 9:1–4
65. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697–703
66. Wermelinger B (2004) Ecology and management of the spruce bark beetle *Ips typographus*-a review of recent research. For Ecol Manage 202:67–82
67. Whitea RL, Kays MB, Friedrich LV, Bene VED (1993) Impact of different statistical methodologies on the evaluation of the in-vitro MICs for *Bacteroides fragilis* of selected cephalosporins and cephamycins. J Antimicrob Chemother 31:57–64
68. Witzgall P, Kirsch P, Cork A (2010) Sex pheromones and their impact on pest management. J Chem Ecol 36:80–100
69. Wood DL (1982) The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. Annu Rev Entomol 27:411–446
70. Xu BB, Liu ZD, Sun JH (2014) The effects of α-pinene on the feeding performance and pheromone production of *Dendroctonus valens*. Entomol Exp Appl 150:269–278
71. Yilmax H, Sezen K, Kati H, Demirbağ Z (2006) The first study on the bacterial flora of the European spruce bark beetle, *Dendroctonus micans* (Coleoptera: Scolytidae). Biologia 61:679–686
72. Zhang L, Sun J, Clarke SR (2006) Effects of verbenone dose and enantiomer on the interruption of response of the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Scolytidae), to its kariomones. Environ Entomol 35:655–660
73. Zhang L, Clarke SR, Sun J (2009) Electrophysiological and behavioral responses of *Dendroctonus valens* (Coleoptera: Curculionidae: Scolytinae) to four bark beetle pheromones. Environ Entomol 38:472–477