Gene 520 (2013) 47–63

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Isolation and expression of cytochrome P450 genes in the antennae and gut of pine beetle *Dendroctonus rhizophagus* (Curculionidae: Scolytinae) following exposure to host monoterpenes

Claudia Cano-Ramírez [a], María Fernanda López [a], Ana K. Cesar-Ayala [a], Verónica Pineda-Martínez [a], Brian T. Sullivan [b], Gerardo Zúñiga [a,*]

[a] Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prolongación de Carpio y Plan de Ayala, Col. Sto. Tomas, México D. F. CP 11340, Mexico
[b] USDA-Forest Service Southern Research Station, 2500 Shreveport Highway, Pineville, LA 71360, USA

ARTICLE INFO

Article history:
Accepted 29 November 2012
Available online 20 December 2012

Keywords:
Cytochrome P450
Scolytines
Bark beetles
Gene expression
Detoxification

ABSTRACT

Bark beetles oxidize the defensive monoterpenes of their host trees both to detoxify them and convert them into components of their pheromone system. This oxidation is catalyzed by cytochrome P450 enzymes and occurs in different tissues of the insect, including the gut (i.e., the site where the beetle's pheromones are produced and accumulated) and the antennae (i.e., the olfactory organs used for perception of airborne defensive monoterpenes as well as other host-associated compounds and pheromones). We identified ten new *CYP* genes in the pine beetle *Dendroctonus rhizophagus* in either antennae or gut tissue after stimulation with the vapors of major host monoterpenes α-pinene, β-pinene and 3-carene. Five genes belong to the CYP4 family, four to the CYP6 family and one to the CYP9 family. Differential expression of almost all of the *CYP* genes was observed between sexes, and within these significant differences among time, stimuli, anatomical region, and their interactions were found upon exposure to host monoterpenes. Increased expression of cytochrome P450 genes suggests that they play a role in the detoxification of monoterpenes released by this insect's host trees.

1. Introduction

Several highly efficient enzyme systems (cytochrome P450, glutathione *S*-transferases and esterases) have evolved in herbivorous insects for metabolizing plant defensive chemicals to less toxic or more soluble compounds (Dowd et al., 1983; Feyereisen, 1999; Guengerich, 1990; see Schuler, 2011 and citations therein). Cytochrome P450 enzymes constitute an ancient multi-gene family that is highly variable, diverse, and widely distributed throughout the phylogenetic scale (Keeling and Bohlmann, 2006; Nelson, 2011; Nelson and Strobel, 1987). Insect cytochrome P450 enzymes carry out aerobic oxidations on a diversity of organic molecules that are endogenous, exogenous, synthetic or natural. Cytochrome P450 enzymes are encoded by microsomal and mitochondrial *CYP* genes and each P450 is assigned to a family (designated by a number) and subfamily (designated by a letter) (Feyereisen, 1999).

For pine bark beetle species that infest living trees, survival and reproductive success depend on their capacity to overcome the defense mechanisms of their hosts (Christiansen et al., 1987; Franceschi et al., 2005). Pines release both constitutive and induced oleoresins into the beetles' mines within the phloem tissue, and these can physically restrict beetle movement and thereby hinder colonization (Franceschi et al., 2005; Hanover, 1975). In addition, monoterpenes present in the resin, including α-pinene, β-pinene, myrcene, limonene and 3-carene, can injure or kill insects through their toxic effects (López et al., 2011; Smith, 1965). Pine bark beetles complete their life cycle entirely within the bark of host trees, and thus both adults and offspring are invariably exposed to high concentrations of these monoterpenes (Wood, 1982).

There is much direct and indirect evidence that bark beetles can oxidize a broad range of known or potentially toxic substrates with cytochrome P450 enzymes, and it is speculated that this capacity may represent an evolved response against host defensive chemicals (see Seybold et al., 2006 and citations therein). On the other hand, some of the pheromone components of many bark beetle species are oxygenated

Abbreviations: aa, amino acid(s); AAI, amino acid sequence identity; AIC, Akaike information criterion corrected; aLRT, approximate likelihood ratio test; BIC, Bayesian information criterion; bp, base pairs; cDNA, DNA complementary to RNA; Cq, quantification cycles; *CYP*, cytochrome P450 gene; dNTP, deoxyribonucleoside triphosphate; EST, expressed sequence tags; EtdBr, ethidium bromide; FAM, 6-carboxyfluorescein; IPTG, isopropyl β-D-thiogalactopyranoside; kDa, kilodalton(s); Km, kanamycin; LB, Luria–Bertani (medium); MGB, Minor Groove Binder; ML, maximum likelihood; MMLV-RT, Moloney Murine Leukemia Virus-Reverse Transcriptase; OBP, odorant binding protein; ODE, odor degrading enzyme; Oligo, oligodeoxyribonucleotide; ORF, open reading frame; PBS, phosphate buffer solution; PCR, polymerase chain reaction; PI, isoelectric point; RCSB, Brookhaven protein data bank; RLM-RACE, RNA ligase-mediated rapid amplification of the 5′ and 3′ cDNA ends; rRNA, ribosomal RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SE, standard error; SDR, dehydrogenase reductase; SRS, substrate recognition sites; *UTR*, untranslated region(s); Xgal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

* Corresponding author. Tel.: +52 55 57296000x62419.
E-mail address: capotezu@hotmail.com (G. Zúñiga).

0378-1119/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2012.11.059

monoterpenes possessing carbon backbones identical to monoterpenes of their host trees. Evidence suggests that P450 enzymes are involved in this pheromone synthesis through both the oxidation of host-derived monoterpene precursors and the transformation of intermediate compounds produced in endogenous metabolic routes (Aw et al., 2010; Huber et al., 2007; Hunt and Smirle, 1988; Vanderwell and Ochlschlager, 1987; White et al., 1979, 1980). Studies combining biochemical, molecular and functional genomics techniques recently demonstrated that cytochrome P450 enzymes encoded by orthologous genes *CYP9T1* and *CYP9T2* in the midgut of male *I. confusus* LeConte and *I. pini* (Say) are able to oxidize myrcene into (4*R*)-(−)-ipsdienol, an aggregation pheromone component produced by males of these species (Figueroa-Teran et al., 2012; Sandstrom et al., 2006, 2008). These results confirmed that these enzymes have a biochemical function associated with the *de novo* biosynthesis of monoterpenoid aggregation pheromone components within the midgut and possibly the fat body of bark beetles (Blomquist et al., 2010).

Dendroctonus rhizophagus Thomas and Bright is endemic to the northern portion of the Sierra Madre Occidental in northwestern Mexico (Mendoza et al., 2011), where it colonizes exclusively seedlings and young saplings (height<3 m) of 11 pine species. This aggressive bark beetle infests both commercial plantations and areas of natural regeneration and has one generation per year. Typically, individual pine tree hosts are killed and colonized by merely one or a few beetle pairs, thus this species lacks the communal mass-attack behavior typical of this genus (Sánchez-Martínez and Wagner, 2009; Thomas and Bright, 1970). Electrophysiological assays have shown that the antennae of *D. rhizophagus* possess a high degree of olfactory sensitivity to host monoterpenes α-pinene, β-pinene and 3-carene (Cano-Ramírez et al., 2012). In addition, oxygenated monoterpenes including *cis*-verbenol, *trans*-verbenol, verbenone, myrtenal and myrtenol have been identified in the midgut and hindgut of this species (Cano-Ramírez et al., 2012), and field tests suggest that *trans*-verbenol may be a sex pheromone in this species (C. Cano-Ramírez, unpublished data).

The purpose of our study was to identify and characterize the expression profile of cytochrome P450 genes in unfed *D. rhizophagus* individuals exposed to racemic α-pinene, (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene, (*S*)-(−)-β-pinene and (+)-3-carene. These compounds were chosen because they produce an attractive behavioral response on this bark beetle (Cano-Ramírez et al., 2012) and because they are the most abundant monoterpenes in the oleoresin of this beetle's principal hosts, *Pinus arizonica* Engelm. and *P. engelmannii* Carr. (Smith, 2000).

We investigated CYP expression specifically in the antennae because studies of other insect systems indicate that antennal P450s function as odor degrading enzymes (ODEs) for pheromones (Maïbèche-Coisne et al., 2004a; Wojtasek and Leal, 1999) and host-plant volatiles (Dickens et al., 1992); these P450s presumably assure signal deactivation by eliminating odorants from the olfactory sensilla following stimulation. Bark beetles depend on their antennae to perceive a diversity of airborne chemicals that mediate host location and discrimination, mate selection and courtship, attack density regulation, and avoidance of interspecific competition (Byers, 1995). Hence antennal P450 enzymes may be necessary for bark beetles to perceive and respond appropriately to their olfactory environment. Further, we studied the gut because its tissues are involved in the detoxification processes of many insects (Kasai et al., 2000; Tartar et al., 2009), and the biosynthesis of pheromones in both *Ips* and *Dendroctonus* bark beetles has been demonstrated to occur in the anterior midgut (see Blomquist et al., 2010 and citations therein).

2. Material and methods

2.1. Insects and treatments for CYP gene diversity

Pre-emergent, unfed adult *D. rhizophagus* were collected directly from naturally infested Arizona pines (*P. arizonica* Engelm.) in the Predio La Laja I, San Juanito, Bocoyna Municipality, Chihuahua, Mexico (27° 55′ 54.9″ N and 107° 35′ 54.6″ W; at 2452 m) in June 2008. The beetles were stored in plastic containers with a moistened paper towel at 4 °C, transported to the laboratory and starved for two weeks at 4 °C.

Five groups of 12 unfed beetles (1:1 sex ratio) were placed in glass Petri dishes (100 mm × 15 mm) lined with dry filter paper and independently exposed to the vapors of racemic α-pinene (98% chemical purity, 1:1 enantiomeric ratio), (*R*)-(+)-α-pinene (98%), (*S*)-(−)-α-pinene (98%), (*S*)-(−)-β-pinene (99%) and (+)-3-carene (90%) for 24 h at 8 °C under conditions of darkness. All compounds were obtained from Sigma Aldrich, St. Louis, MO, USA. A neat monoterpene 0.5 mM (López et al., 2011) was applied to the filter paper immediately prior to placement of beetles into the dish (not on the monoterpene application area), and then the dish was closed and sealed with Parafilm, to minimize the diffusion of these compounds out of the dishes.

Immediately following terpene exposure, beetles (n = 60) were immersed in phosphate buffer solution (PBS) and dissected. The antennae were removed directly from the insects, and the gut was dorsally extracted after the elytra and wings were removed. The gut was separated from the fat body and the Malpighian tubules and sectioned into foregut, midgut and hindgut (after Díaz et al., 1998). The antennae and gut sections from insects of each treatment were separately placed into Eppendorf vials with 0.2 μl of Trizol® (Invitrogen Corp., Carlsbad, CA, USA) and macerated completely using a sterile pestle. Then each vial was topped with 1 ml Trizol and stored at −80 °C for posterior RNA extraction.

2.2. RNA isolation and cDNA synthesis

Total RNA isolation of the antennae and gut regions was conducted following the protocol provided in the Trizol® kit. The integrity of the total RNA was visually assessed on a denaturing formaldehyde 1% agarose gel that was stained with 10 μg/ml ethidium bromide (EtdBr) at room temperature (Sambrook et al., 1989). The cDNA was synthesized from 1 μg of total RNA following the protocol provided with the MMLV-RT Moloney Murine Leukemia Virus Reverse Transcriptase enzyme (Invitrogen) kit and using the Oligo (dT) 12–18 primer (GE Healthcare, Buckinghamshire, UK).

2.3. Degenerate polymerase chain reaction, cloning and sequence analyses

The synthetized cDNA from the antennae, foregut, midgut and hindgut from each monoterpene treatment was used as the template in all of the PCR. Pairs of degenerate primers (Supplementary material Table 1) were used to screen the putative P450 cDNA from the CYP4 (clade 4), CYP6 (clade 3) and CYP9 (clade 3) families, which are involved in detoxification processes (Feyereisen, 2006). We did not look for CYP2 genes because this clade is implicated in reactions during insect molting and metamorphosis (Namiky et al., 2005). The CYP4 genes were amplified following Snyder et al. (1996), except for the T_m, which was 49 °C; the CYP6 and CYP9 genes were amplified following Dunkov et al. (1996). PCR was performed in a Biometra T Thermocycler (Biometra, Göttingen, Germany) using 30 μl reactions containing 2 μl cDNA, 1× Taq polymerase buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.33 μM of each primer, and 1.5 U of recombinant *Taq* polymerase (Invitrogen Life Technologies, Sao Paulo, Brazil). The reaction conditions were an initial 5-min step at 94 °C followed by 35 cycles of 60 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C, and a final extension for 5 min at 72 °C. The PCR products were visualized on 1% agarose gels stained with 10 μg/ml EtdBr and compared with a 100-base pair (bp) DNA ladder (Gibco, BRL, Gaithersburg, MD, USA). The amplicons were then purified with the GFX PCR DNA kit (Amersham Biosciences, UK) and/or Gel Band Purification kit (GE Healthcare), and each reaction product was cloned using the pDrive Cloning® Kit (Qiagen, Valencia, CA, USA).

The cloning reactions were transformed in chemically competent DH5α cells of *Escherichia coli* (Sambrook et al., 1989). The transformants

(blue-white colonies) were selected on Km/LB/Xgal/IPTG plates. Ten colonies of each treatment for each gene ($n_{total} = 320$) were grown overnight in Km/LB broth and the plasmid DNA was extracted by the alkaline lysis method (Sambrook et al., 1989). The insertion was verified by a simple restriction with the *Eco*R1 enzyme (Invitrogen) and visualized on 1% agarose gels. The recombinant plasmids were purified for sequencing using the QIAquick® PCR Purification Kit (Qiagen). A total of 230 clones with the inserts were sequenced using a CEQ 8000 Genetic Analysis System and Quick Start Master Mix® 2× (Beckman Coulter, Fullerton, CA).

2.4. Identification of CYP variants

Fragments of ≈400 to 500 bp were subjected to Blastx searches against the NCBI database. Putative P450 gene sequences were manually edited with SEAVIEW v 4.2.5 (Gouy et al., 2010) to achieve positional homology with the variants that matched. The deduced amino acid sequences were obtained with the ExPasy Translate Tool (http://www.expasy.org/tools/dna.html) and were then once more subjected to a BLASTp search (Altschul et al., 1990) against the NCBI database. A multiple sequence alignment of the P450 proteins was conducted with CLUSTAL X v 2.0.10 (Thompson et al., 1997) using default parameters. To recognize the different CYP variants that were expressed in the antennae and gut tissue in 239 clones, a phylogenetic reconstruction analysis was performed using maximum likelihood (ML) with LRT-PHYML (Guindon and Gascuel, 2003). The CYP topology was used to identify groups, but not to establish phylogenetic relationships. Before the ML analysis, we determined an appropriate model of protein evolution and model parameters using both the Akaike Information Criterion Corrected (AIC = 10447.15, −lnL = −4714.58) and the Bayesian Information Criterion (BIC = 11925.9, −lnL = −4714.58) tests, implemented in PROTEST v 2.4 (Abascal et al., 2005). Both tests supported the LG + I + G model (Le and Gascuel, 2008) with an estimated proportion of invariable sites (I = 0.038) and gamma shape of 2.513. We chose to optimize the topology of the tree rather than the branch length. To estimate the support for each node, the approximate likelihood ratio test (aLRT, Anisimova and Gascuel, 2006) with the Shimodaira–Hasegawa-like procedure option was used. As outgroup sequences for this analysis, we used *Tribolium castaneum* Herbst (CYP4: accession no. AAF70496), *I. paraconfusus* (CYP4: accession no. ABF06544, ABF06546, ABF06550, ABF06553), *Dendroctonus ponderosae* Hopkins (CYP4: accession no. AFI45016, AFI45017, AFI45019, AFI45021, AFI45022), *Helicoverpa armigera* (Hübner) (CYP4: accession no. AA033078), *D. ponderosae* (CYP6: accession no. AFI45024, AFI45025, AFI45026, AFI45027, AFI45036, AFI45040, AFI45041), *An. minimus* (CYP6: accession no. ANN05727), *D. ponderosae* (CYP9: AFI45045 and AFI45046) and *I. confusus* (CYP9: ACK37844). To determine whether the sequences that were included within the main groups' output from the phylogenetic analysis actually belonged to the same CYP family, the identity percentages among the amino acid sequences were estimated using MatGAT v. 2.01 (Campanella et al., 2003). Our cut-off for considering one sequence within the same family was <40%, an upper limit similar to those established by the International P450 Nomenclature Committee (Nelson et al., 1996).

2.5. End sequence determination and cloning of full-length cDNAs

The complete sequences of CYP genes identified in the phylogenetic analysis were achieved with the RNA ligase-mediated rapid amplification of the 5′ and 3′ cDNA ends (RLM-RACE) kit (Invitrogen). The total RNA of both the antennae and the gut of unfed bark beetles stimulated for 24 h with racemic α-pinene was obtained, following the protocol described in the RiboPure® Kit (Ambion, Inc., Austin, TX, USA); its integrity was checked on denaturing formaldehyde 1% agarose gels. The total RNA quantification was done by spectrophotometry with a Beckman Coulter TU800 (Beckman Coulter, Inc., Brea CA, USA). The purity was estimated by mean of relation A260/A280 (μg/ml = A260 × dilution factor × 40). Partial sequences were used in the primer design and PCR was carried out following the protocol described in the RLM-RACE kit. The amplicons were purified, cloned and sequenced as previously described. The complete sequences were compared using a BLASTp search with those deposited in GenBank (Altschul et al., 1990).

To avoid chimera sequences, we designed specific primers (Supplementary material Table 1) based on the complete sequences obtained for each CYP gene with RACE; these specific primers, were used to amplify the complete DNA for each gene. Amplification reactions were carried out in 25 μl volumes containing: 2 μl cDNA from a 1:10 dilution, 1× high fidelity PCR buffer, 2.0 mM $MgSO_4$, 0.2 mM dNTPs, 0.33 μM of each primer (Invitrogen), and 1.25 U of Platinum® *Taq* DNA polymerase high fidelity (Invitrogen). Touchdown PCR was performed as follows: 2 min at 94 °C, 5 cycles of 30 s at 94 °C, 2 min at 72 °C, 5 cycles of 30 s at 94 °C, 2 min at 70 °C, 35 cycles of 60 s at 94 °C, 60 s at 68 °C, 60 s at 68 °C, and then a final extension of 10 min at 68 °C. PCR products of ≈1500 bp were visualized on 1% agarose gels, purified, cloned and sequenced in both strands as previously described. The deduced amino acid sequences were submitted to the P450 nomenclature committee and names assigned based upon their criteria for the classification of CYP genes (D. Nelson Department of Molecular Sciences, University of Tennessee, personal communication). All sequences were deposited in GenBank (accession no. HQ113133–HQ113142).

2.6. Analysis of full length cytochrome P450 sequences

The molecular mass (kDa) and isoelectric point (PI) of each sequence were determined using the ProtParam program (Gasteiger et al., 2005). The ten fully sequenced CYP genes were grouped in three alignments. For each alignment, the sequences from *I. paraconfusus* (*CYP4G27*: accession no. ABF06553), and *T. castaneum* (*CYP6BK17*, XP_969813 and *CYP9Z4*, NP_001164248) were selected as a representative set from GenBank. Crystal structure data for the mammalian P450 CYP3A4 (PDB code 1TQN) protein were downloaded from the RCSB (Brookhaven protein data bank) website (http://www.rcsb.org/) and used as the template for the secondary structure. The program ESPript (Gouet et al., 2003) was used for the assignment of secondary structure elements onto the corresponding aligned sequences, and the substrate recognition sites were manually indicated based on the CYP3A4 enzyme information. All putatively functional *D. rhizophagus* P450 proteins were checked for likely sub-cellular localization using the TargetP program (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al., 2000) and WoLF PSORT (http://wolfsport.org/) (Horton et al., 2007) with the default parameters.

2.7. Insects and treatments for RT-qPCR

Pre-emerged insects of *D. rhizophagus* were collected as described above. Beetles were housed in vented plastic containers with moistened paper at 4 °C for up to 15 days before use. Insects were sexed according to the shape of the seventh abdominal tergite (Lyon, 1958). Ten groups (5♀and 5♂) each of them integrated by 20 unfed beetles were placed in glass Petri dishes and stimulated independently with 100 μl of the following monoterpenes: 1 M of α-pinene (1:1 racemic mix), (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene (*S*)-(−)-β-pinene and (+)-3-carene. Ten insects of each group and treatment were dissected at 8 and 24 h after stimuli. Two groups additional of ten non-stimulated insects by each sex were dissected at beginning of the experiment (time zero) and used as control groups. The expression of all genes was referred to this time zero. The antennae and each gut sections of the insects were removed and processed as previously described.

2.8. RNA isolation and cDNA synthesis for expression analyses

The total RNA isolation of the antennae and gut sections were made following the protocol described in the RiboPure® Kit and its integrity was checked in 1% agarose gels. The cDNA synthesis was done using the protocol described in the High Capacity RNA to Cdna® Kit (Applied Biosystems, Foster City CA, USA) using 2 μg total RNA in a 20 μl final reaction volume. The synthesis cDNA program was: 37 °C for 60 min and 95 °C for 5 min. A non-reverse transcription assay was performed to evaluate the DNA absence in the RNA extraction. The cDNA was stored to −20 °C.

2.9. RT-qPCR

For each target gene, two primers and a probe were designed by Applied Biosystems (Supplementary material Table 2). All probes were deposited in the NCBI Probe database (Accession no. 27564573-27564583). The TaqMan® hydrolysis probes were labeled in the 5′ end, with reporter dye 6-carboxyfluorescein (FAM) and in the 3′ end was joint to a quencher non-fluorescent molecule group MGB (Minor Groove Binder). The reaction was carried out on the Step One® TM Real-Time (Applied Biosystems) under the following conditions: each PCR reaction contained 20× primer and probes with a final concentration of 900 nM for each primer, 250 nM for each TaqMan probe, TaqMan® Universal Master Mix II (Applied Biosystems) and 5 μl of the diluted cDNA sample in a final volume of 20 μl. All samples were place in a Fast Optical 48-well reaction plate (MicroAMO™, Applied Biosystem). The manufacturer's standard amplification conditions were used: 50 °C for 2 min, 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 60 s. PCR contaminations were not detected in the no template control (NTCs). The experiment was replicated three times (biological replicates) each of them with three technical replicate.

To estimate the qPCR efficiency and validation for each gene, a linear regression analysis was performed between the mean values of quantification cycles (Cq) of different dilutions (1.0, 0.2, 0.04, 0.008, 0.0016 μg/μl) of cDNAs and its initial concentration. These dilutions are made from a cDNAs pool and 5 μl of each dilution are taken as qPCR template, which is performed for each gene three times. The PCR efficiency was estimated with the relation: efficiency $= (10^{-1/\text{slope}} - 1) \times 100$, where the efficiency value was of -3.32 ± 0.02. The PCR validation was estimated directly from R^2 values, which were >0.96. All amplicons were visualized in 1% agarose gel electrophoresis to evaluate their specificity.

2.10. Reference gene validation experiment

To determine the most stably transcribed reference gene, a validation of cytoplasmic β-actin, 18S rRNA, and *CYP4G55v1* from *D. rhizophagus* was performed. The genes β-actin and 18S rRNA primers were taken from Rongnoparut et al. (2003) and McKenna et al. (2009) respectively. PCR products of ≈500 bp were visualized on 1% agarose gels, purified, cloned and sequenced as previously described. For cytoplasmic β-actin gene new primers were designed (Supplementary material Table 1), and a PCR product of ≈200 bp was obtained and tested in semi quantitative-PCR tests with negative results. On the other hand, for 18S rRNA gene, two primers and a probe were designed by Applied Biosystems (Supplementary material Table 2) and used in RT-qPCR. Ct values from 18S rRNA and all of the *D. rhizophagus* CYP4, CYP6 and CYP9 were analyzed with the geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) algorithm in the Genex Light software package (http://www.multid.se). *CYP4G55v1* was the most stable expression under our conditions and was used as a reference gene to normalize the data, except when the transcript levels of *CYP4G55v1* was analyzed, wherein we used the 18S rRNA (the next most stably transcribed gene).

2.11. Statistical analysis

Relative expression values for all genes were determined using the Ct ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2008) and analyzed with Microsoft Excell 2010 (v.14.0.61295000). Outlier values identified by a PCR machine were excluded from our analysis. To evaluate significant differences in the expression for each gene $2^{-\Delta\Delta CT}$ values transformed at $\log_{10}+1$ were subjected to one-way ANOVA to know if the expression gene was different between sexes. If significant differences were found, then a three-way ANOVA was performed by sex to assay the expression of gene among anatomical regions (antennae, foregut, midgut and hindgut), stimuli (α-pinene (1:1 racemic mix), (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene (*S*)-(−)-β-pinene and (+)-3-carene) and time (0 h, 8 h and 24 h). A Tukey's test was performed to know where the differences among these factors occur. The $2^{-\Delta\Delta CT}$ values and standard error (SE) were transformed at $\log_2$ for their graphing. All statistical analyses were performed with SigmaStat 3.5 software.

3. Results

3.1. Identification of cytochrome P450 genes

Ten CYP gene groups (five CYP4, four CYP6, and one CYP9) were found in the ML-phylogenetic analysis with putative cytochrome P450 partial gene sequences from 239 clones (Fig. 1). All groups had strong nodal support values (aLRT > 70%) (Fig. 1). The amino acid sequence identity (AAI) among sequences of the same group was high (>85%) and variable with respect to the reported sequences (27.4%<AAI<96.0%; higher match score in GeneBank, NCBI) (Supplementary material Table 3).

BLAST search analyses indicated that the CYP genes expressed in the antennae and gut regions of *D. rhizophagus* resemble members of the CYP4 (clade 4), CYP6, and CYP9 (clade 3) families in other insects (Supplementary material Table 3). Genes from the CYP4 family shared the higher AAI with variants from the beetles *Dendroctonus ponderosae* Hopkins, *Tribolium castaneum* and *Ips paraconfusus*; and genes from the CYP6 and CYP9 families with variants from *D. ponderosae*.

3.2. Full-length of CYP genes

The full-length of the genes varied from 1581 bp (*CYP4BD5v1*) to 1902 bp (*CYP9Z2Dv1*) and their open reading frame (ORF) varied between 1488 bp (*CYP4BQv1*) and 1686 bp (*CYP4G55v1*) (Table 1). All ORFs encoded about 500 amino acids (aa). The CYP genes were flanked by 5′ and 3′ UTRs (untranslated regions), whose length varied from 24 to 90 bp and 24 to 225 bp, respectively. The predicted molecular mass varied from 57 (*CYP4BQ1v1*) to 64 kDa (*CYP4G55v1*), and the isoelectric point varied from 6.75 (*CYP4G56v1*) to 9.2 (*CYP6DG1v1*) (Table 1). With the exception of the P450 enzyme encoded by the *CYP4G55v1*, the predicted sub-cellular location of the P450 proteins showed that they putatively code for a typical microsomal signal peptide of approximately twenty hydrophobic residues that are likely membrane anchors in the endoplasmic reticulum. In contrast, the predicted sub-cellular location for the P450 enzyme encoded by the *CYP4G55v1*, suggests a cytoplasmic location (Table 1).

The complete amino acid sequences showed the typical conserved P450 domains, including the heme-binding region (FXXGXRXCXG) near the C-terminal end, the PERF domain (PXRX) and the K-helix (EXXR) (Figs. 2–4). The alignment and comparison of the deduced amino acid sequences of *D. rhizophagus* with respect to the *CYP3A4* sequence from mammal, the *CYP4G27* sequence from *I. paraconfusus*, and the *CYP6BK17* and *CYP9Z4* sequences from *T. castaneum* allowed the identification of six putative substrate recognition sites (SRSs), which are located in regions with variable structural elements. Based on the *CYP3A4* secondary structure, 18-α helices and 9-β sheets were recognized (Figs. 2–4). The AAI varied from 44.9% (*CYP6DG1v1*) to 96.8% (*CYP4G56v1*) with respect to the cytochrome P450 enzymes

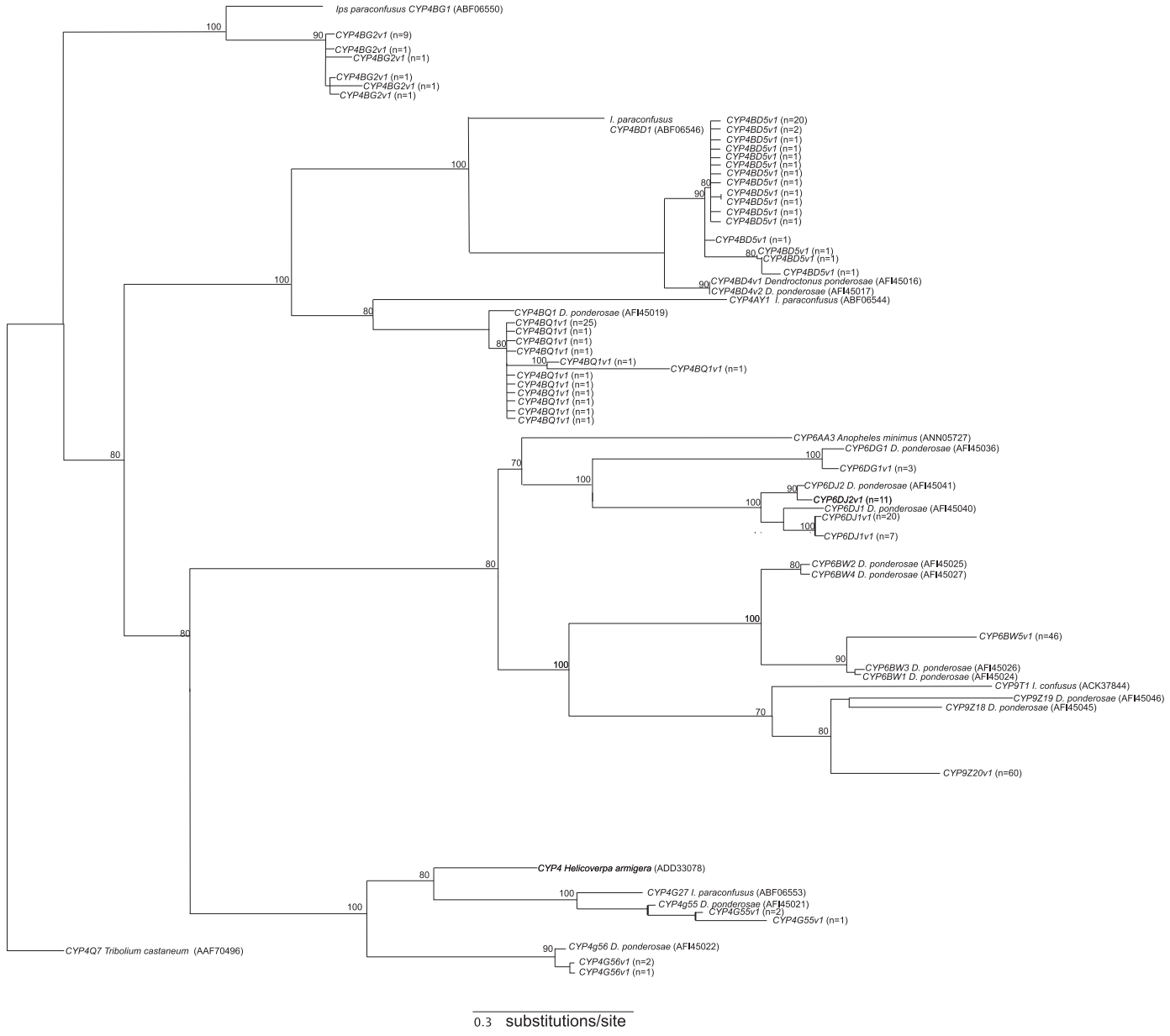


Fig. 1. Maximum-likelihood tree of cytochrome P450 genes from *D. rhizophagus* based ≈166 amino acid sequences using the LG + I + G model with an estimated proportion of invariable sites (I = 0.038). *T. castaneum* (CYP4Q7), *I. paraconfusus* (*CYP4AY1*, *CYP4BD1*, *CYP4BG1*, *CYP4G27*), *I. confusus* (*CYP9T1*), *D. ponderosae* (*CYP4BD4v1*, *CYP4BD4v2*, *CYP4BQ1*, *CYP4g55*, *CYP4g56*, *CYP6DJ1*, *CYP6DJ2*, *CYP6DG1*, *CYP6BW1*, *CYP6BW2*, *CYP6BW3*, *CYP6BW4*, *CYP9Z18*, *CYP9Z19*), *H. armigera* (*CYP4G9*) and *An. minimus* (*CYP6AA3*) were used as the reference sequences. The accession numbers of GenBank sequences are shown in brackets. The (n) indicates the number of clones obtained for each *CYP* gene, and the branch support and aLRT values are shown at the nodes.

from *D. ponderosae*, *I. paraconfusus* and *T. castaneum* (Supplementary material Table 4).

3.3. Quantitative expression of CYP genes

CYP4BD5v1 gene: Statistically significant differences in the transcript expression levels were found between sexes of *D. rhizophagus* (one-way ANOVA, $F = 413.57$, $df = 1$, $p < 0.001$). The females expressed the gene in all anatomical regions with some stimuli and time (Figs. 5A, C, E and G) and the males mainly in the foregut with all monoterpenes and enantiomers at 8 and 24 h (Fig. 5D), also this gene were expressed in the antennae at 24 h with (+)-3-carene. Based on this result, a three-way ANOVA (Tables 2 and 3) and Tukey tests corresponding (Table 4) for each sex revealed significant differences in the expression levels of this gene among stimuli, region, time and interactions among these factors. *CYP4BQ1v1* gene: Sexes showed significant differences in the transcript expression levels (one-way ANOVA, $F = 14.39$, $df = 1$ $p < 0.001$). The females expressed the gene in the foregut at 24 h with racemic α-pinene, and with all stimuli in the midgut at any time, except with (*R*)-(+)-α-pinene enantiomer (Figs. 5C and E). The males only expressed this gene in the antennae at 24 h with (+)-3-carene, and with α-pinene at 8 h, (*S*)-(−)-α-pinene at 24 h, and (+)-3-carene at 8 and/or 24 h in the hindgut (Figs. 5B and H). Likewise, significant differences in the expression levels of this gene among stimuli, region, time and interactions among these factors were shown for each sex by means of a three-way ANOVA (Tables 2 and 3) and Tukey test correspondingly (Table 4). *CYP4BG2v1* gene: Statistically significant differences in the expression gene were found between sexes (one-way

Table 1
Physicochemical characteristics predictive of cytochrome P450 enzymes encoded by *CYP* genes isolated from antennae and gut samples from *Dendroctonus rhizophagus*.

Full length CYP name	ORF size (bp)	Protein flanked in 5′ 3′ (bp)	Molecular mass[a] (kDa)	PI[a]	Signal peptide prediction[b,c]
CYP4BQ1v1	1488	48–91	57,352.6	8.51	SP 0.952 mTP 0.133 others 0.011
CYP4BD5v1	1512	45–24	57,648.7	9.05	SP 0.954 mTP 0.027 others 0.067
CYP4BG2v1	1503	52–101	57,836.1	6.99	SP 0.944 mTP 0.37 others 0.042
CYP4G55v1	1686	42–128	64,434.4	8.28	SP 0.403 mTP 0.034 others 0.627
CYP4G56v1	1650	42–51	63,639.3	6.75	SP 0.715 mTP 0.22 others 0.542
CYP6DJ1v1	1500	27–67	57,433.6	9.05	SP 0.985 mTP 0.11 others 0.053
CYP6DJ2v1	1521	31–225	58,344.5	8.82	SP 0.980 mTP 0.17 others 0.047
CYP6BW5v1	1515	37–159	57,683.8	8.43	SP 0.887 mTP 0.83 others 0.095
CYP6DG1v1	1521	24–72	58,765.0	9.2	SP 0.965 mTP 0.017 others 0.053
CYP9Z20v1	1596	90–216	61,387.2	8.65	SP 0.914 mTP 0.069 others 0.068

SP = secretory pathway signal peptide and mTP = mitochondrial targeting peptide.
[a] As predicted by ProtParam (Gasteiger et al., 2005).
[b] As predicted by TargetP (Emanuelsson et al., 2000).
[c] As predicted by WoLF PSORT (Horton et al., 2007).

ANOVA, $F=22.4$, $df=1$, $p<0.001$). The gene was expressed by the females mainly in the foregut with all stimuli in any time (Fig. 5C), the expression in the males occurred in the antennae with all stimuli mainly at 8 h and a slight expression in the foregut only with racemic α-pinene and (*S*)-(−)-α-pinene at 8 h (Figs. 5B and D). Significant differences in the expression levels of this gene among stimuli, region, time and interactions among these factors were shown for each sex by means of three-way ANOVA (Tables 2 and 3) and Tukey test correspondingly (Table 4). *CYP4G56v1* gene: Sexes showed significant differences in the expression gene (one-way ANOVA, $F=10.04$, $df=1$ $p=0.002$). Both sexes expressed the gene slightly in the foregut and hindgut with all stimuli and any time (Figs. 5C, D, G and H) and in the antennae only by the males at 8 and 24 h with (*R*)-(+)-α-pinene and (*S*)-(−)-β-pinene, respectively (Fig. 5B). Significant differences in the expression levels of this gene among stimuli, region, except time and interactions among these factors were shown for each sex by means of three-way ANOVA (Tables 2 and 3). The differences among factors by means of corresponding Tukey test are shown in the Table 4. *CYP4G55v1* gene: No significant differences between sexes were found in the expression gene (one-way ANOVA, $F=0.232$, $df=1$, $p=0.630$). The gene was inhibited in all regions, stimuli and time in both sexes (Fig. 5), except in the foregut from females with racemic α-pinene at 8 h (Fig. 5C), and in the male's antenna and hindgut with (*S*)-(−)-α-pinene at 24 h and racemic α-pinene at 8 h, respectively (Figs. 5B and H). Based on the one-way ANOVA results, female and male data were clustered for three-way ANOVA and Tukey test. Significant differences in the expression levels of this gene were found with three-way ANOVA among regions ($F=73.8$, $df=3$, $p<0.001$), stimuli ($F=94.8$, $df=5$, $p<0.001$), time ($F=555.8$, $df=2$, $p<0.001$) and the interactions: region∗stimuli ($F=5.1$, $df=15$, $p<0.001$), regions∗time ($F=24.6$, $df=6$, $p<0.001$), stimuli∗time ($F=27$, $df=10$, $p<0.001$) and region∗stimuli∗time ($F=2$, $df=30$, $p<0.001$). The differences among factors by means of corresponding Tukey test are shown in the Table 4. *CYP6BW5v1* gene: Significant differences were found between sexes in the expression gene (one-way ANOVA, $F=26.4$, $df=1$ $p<0.001$). This gene was expressed in all anatomical regions in both sexes in any stimuli and time (Figs. 6A–H). Significant differences in the expression levels of this gene among stimuli, region, time and interactions among these factors were shown for each sex by means of a three-way ANOVA (Tables 2 and 3) and Tukey tests correspondingly (Table 4). *CYP6DG1v1* gene: No significant differences between sexes were found in the expression gene (one-way ANOVA, ($F=1.34$, $df=1$, $p=0.247$)). This gene was expressed in all anatomical regions in both sexes with some stimuli and time (Figs. 6A–H). Based on the one-way ANOVA results, data for both sexes were grouped for three-way ANOVA and Tukey test. Significant differences in the expression levels of this gene were found with three-way ANOVA among regions ($F=248.6$, $df=3$, $p<0.001$), stimuli ($F=30.7$, $df=5$, $p<0.001$), time ($F=151.3$, $df=2$, $p<0.001$) and the interactions: region∗stimuli ($F=23.8$, $df=15$, $p<0.001$), regions∗time ($F=83.2$, $df=6$, $p<0.001$), stimuli∗time ($F=47.4$, $df=10$, $p<0.001$) and region∗stimuli∗time ($F=15.5$, $df=30$, $p<0.001$). The differences among factors by means of corresponding Tukey tests are shown in Table 4. *CYP6DJ1v1* gene: Sexes showed significant differences in the expression gene (one-way ANOVA, $F=30.42$, $df=1$, $p<0.001$). Female gene expressed slightly in the foregut with racemic α-pinene at 8 h and 24 h (Fig. 6C), and with racemic α-pinene and (*S*)-(−)-β-pinene in the midgut at 24 and 8 h, respectively (Fig. 6E). The males expressed the gene in the midgut with all stimuli at any time (Fig. 6F), and with racemic α-pinene in the antenna and hindgut at 24 h (Figs. 6B and H). Significant differences in the expression levels of this gene among stimuli, region, time and interactions among these factors by sex were shown by means of three-way ANOVA (Tables 2 and 3) and Tukey test correspondingly (Table 4). *CYP6DJ2v1* gene: Significant differences were found in the expression gene between sexes (one-way ANOVA, $F=16.93$, $df=1$, $p<0.001$). The gene was expressed in all regions analyzed in both sexes with some stimuli and time (Figs. 6A–H), however, the males over-expressed the gene mainly in the antenna and hindgut with all stimuli at any time. Significant differences in the expression levels of this gene among stimuli, region, time and interactions among these factors by sex were shown by means of three-way ANOVA (Tables 2 and 3). The differences among factors by means of corresponding Tukey tests are shown in the Table 4. Lastly *CYP9Z20v1* gene: Sexes showed significant differences in the gene expression (one-way ANOVA, $F=12.66$, $df=1$, $p<0.001$). The gene was only expressed in the gut regions in both sexes with any stimuli and time, nevertheless, the gene was expressed in the male's antenna with racemic α-pinene. Significant differences among stimuli, region, time and interactions among these factors by sex were shown by means of three-way ANOVA (Tables 2 and 3) and Tukey test correspondingly (Table 4).

4. Discussion

In this study, we report ten new bark beetle cytochrome P450 belonging to the families CYP4, CYP6 and CYP9, and we reported the expression of specific CYP genes from antennae of the species of *Dendroctonus*. The phylogenetic analysis of all clones sequenced showed a high diversity within each gene, particularly within of *CYP4BQ1v1*, *CYP4BD5v1*, and *CYP4BG2v1* genes, suggesting that serial duplication events had occurred through their evolution (Fig. 1) (Hahn, 2009).

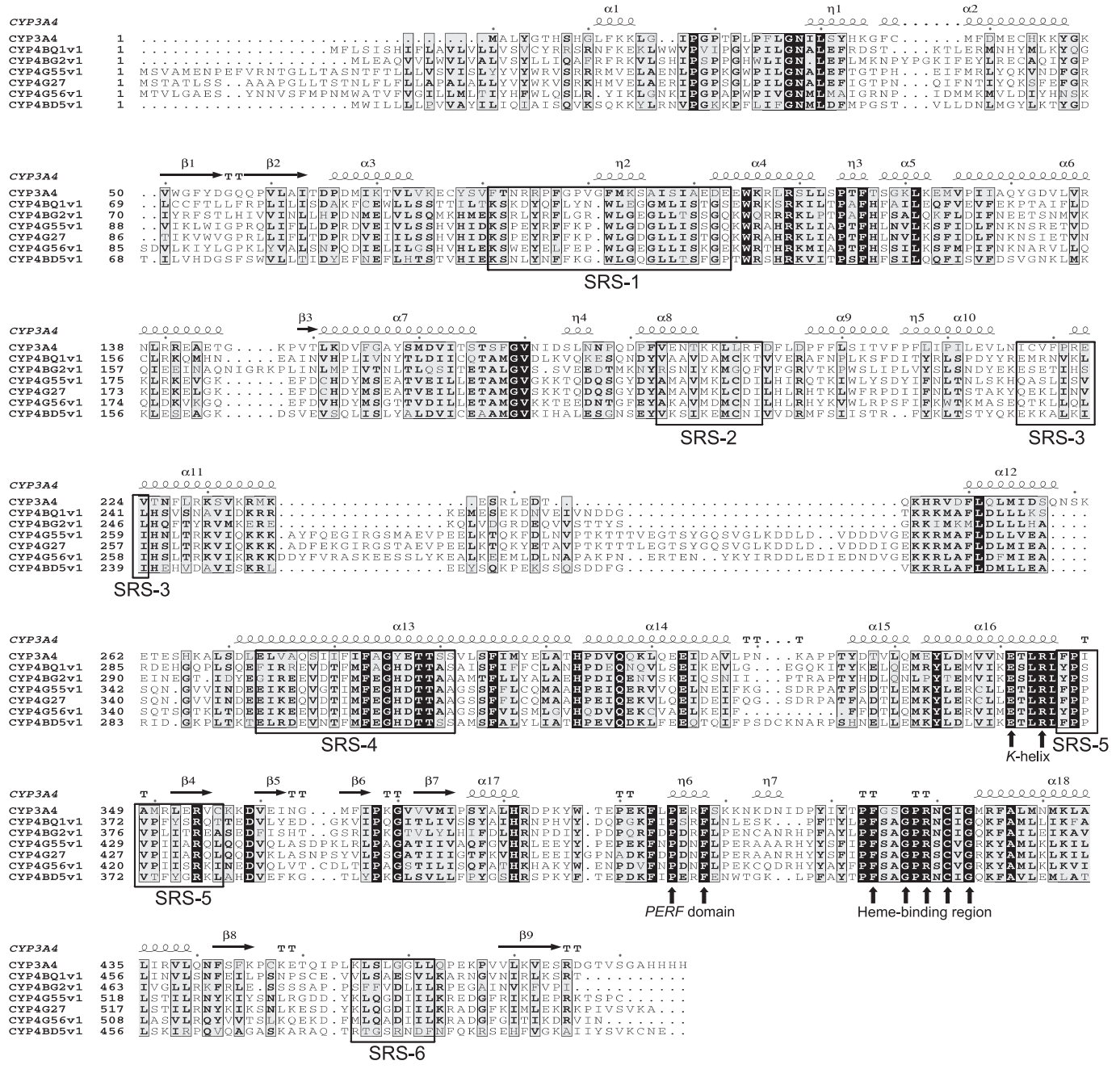


Fig. 2. Multiple sequence alignment and secondary structure element assignment. The alignment included *CYP4BQ1v1* (accession no. HQ113133), *CYP4BG2v1* (HQ113135), *CYP4BD5v1* (HQ113134), *CYP4G55v1* (HQ113136) and *CYP4G56v1* (HQ113137) from *D. rhizophagus*, the predicted *CYP4G27* from *I. paraconfusus* (accession no. ABF06553) and the mammalian CYP3A4 protein sequence. Substrate recognition sites (SRSs) 1–6 were manually determined. The heme-binding region (FXXGXRXCXG), PERF domain (PXRX) and K-helix (EXXR) are indicated with arrows. The alpha helices are marked as alpha or beta, based on the automatic assignment according to the template of the CYP3A4 protein structure in the program ESPript.

The CYP genes we isolated from *D. rhizophagus* exhibited AAI > 70% with cytochrome P450s identified in the transcriptome of mountain pine beetle *D. ponderosae* (Keeling et al., 2012) (Supplementary material Table 3), except the *CYP4BG2v1* that not was identified or reported in *D. ponderosae*. This gene showed an AAI ≈ 58% with the bark beetle, *Ips paraconfusus* (Huber et al., 2007). The difference in the number of genes recognized between these bark beetle studies is likely explained by the fact that Keeling et al. (2012) analyzed larvae, pupae and adults under different stimuli such as racemic juvenile hormone, monoterpenes and feeding with phloem from pine host. On the other hand, Huber et al. (2007) analyzed whole insects rather than specific tissues; thus their samples included additional anatomical regions (such as the fat body and Malpighian tubules) where CYP gene expression has been reported previously. Furthermore, the insects were feeding on the phloem of ponderosa pine, *Pinus ponderosa* Lanier and thus had presumably been exposed to a greater number of constituent host compounds than in the present study.

Another difference between the studies was the type of CYP genes identified. Twelve genes from the CYP4 family and one of the CYP9 family were reported in *I. paraconfusus*, whereas in *D. rhizophagus*

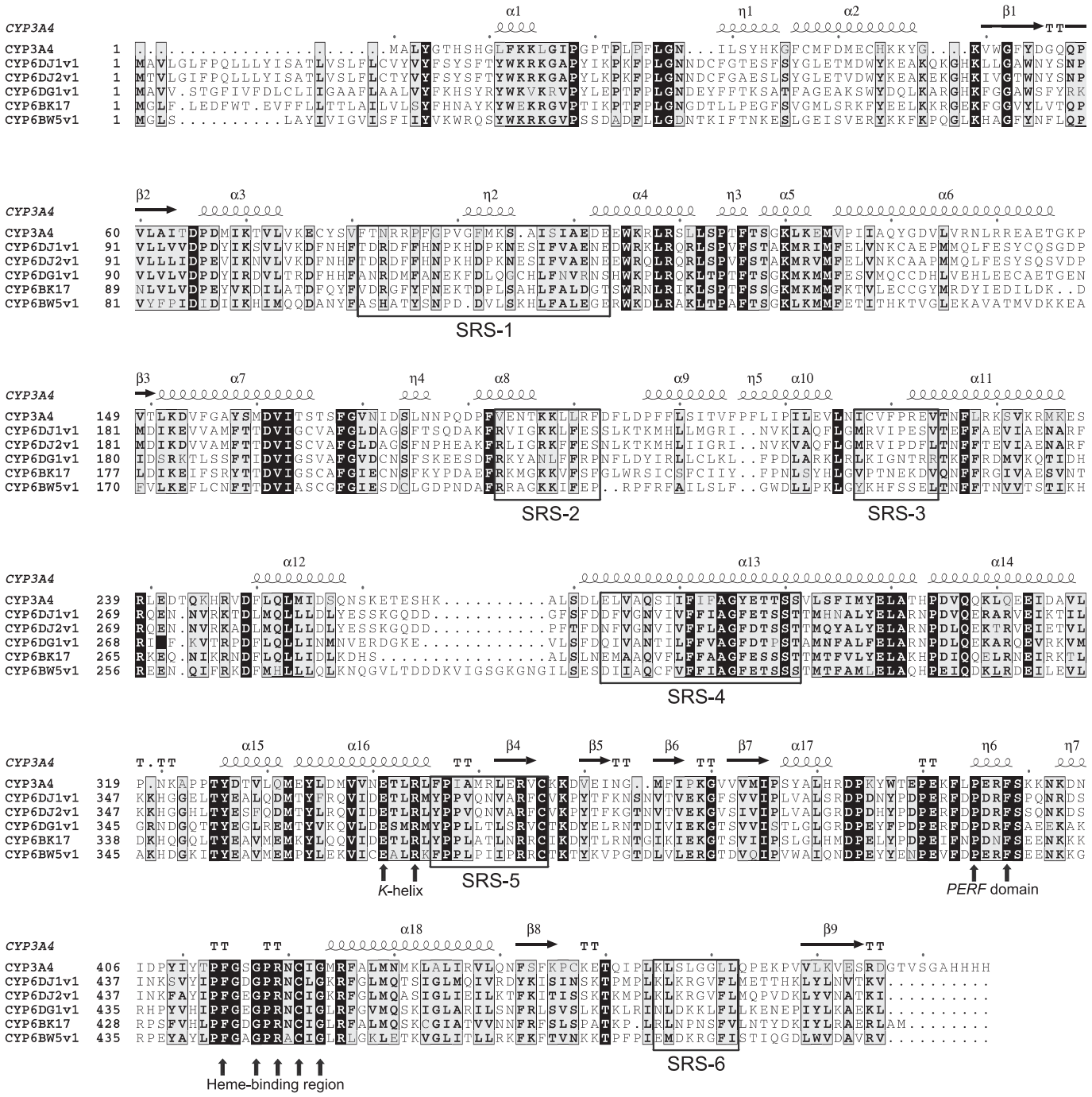


Fig. 3. Multiple sequence alignment and secondary structure elements assignment. The alignment included *CYP6DJ1v1* (accession no. HQ113138), *CYP6DJ2v1* (HQ113138), *CYP6DG1v1* (HQ113141), *CYP6BW5v1* (HQ113140) from *D. rhizophagus*, the predicted *CYP6BK17* from *T. castaneum* (accession no. XP_969813) and the mammalian CYP3A4 protein sequence. Substrate recognition sites (SRSs) 1–6 were manually determined. The heme-binding region (FXXGXRXCXG), PERF domain (PXRX) and K-helix (EXXR) are indicated with arrows. The alpha helices are marked as alpha or beta based on the automatic assignment according to the template of the CYP3A4 protein structure in the program ESPript.

we isolated five genes from CYP4, four from CYP6, and one from the CYP9 families. A study of functional genomics of the midgut and fat body of the mountain pine beetle *D. ponderosae* identified representatives of tentative genes for CYP4 and CYP6—but did not mentioned CYP9—families in expressed sequence tags (ESTs) after the insects were stimulated with juvenile hormone (Aw et al., 2010). Recently, the transcriptome of *D. ponderosae* reported 78 cytochrome P450, of which 47 were full-length and the others partial sequences. The full-lengths were 34 from the CYP3 (6 and 9 families) clade, and 9 from the CYP4 clade (Keeling et al., 2012).

The identification and expression of the genes *CYP4BD5v1* (♀, ♂), *CYP4BQ1v1* (♂), *CYP4BG2v1* (♂), *CYP4G56v1* (♂), *CYP6BW5v1* (♀, ♂), *CYP6DG1v1* (♀, ♂), *CYP6DJ2v1* (♀, ♂) and *CYP9Z20v1* (♂) in *D. rhizophagus* antennae suggests, that cytochrome P450 enzymes might be differentially induced by monoterpenes from the moment of host perception, regardless of whether these compounds, alone or blended, have an effect of attraction or repulsion on the beetles (Figs. 5–7). In other insects, such as the fruit fly *Drosophila melanogaster* Meigen, the pale brown chafer *Phyllopertha diversa* Waterhouse, the tobacco hornworm *Manduca sexta* L, and the cabbage armyworm

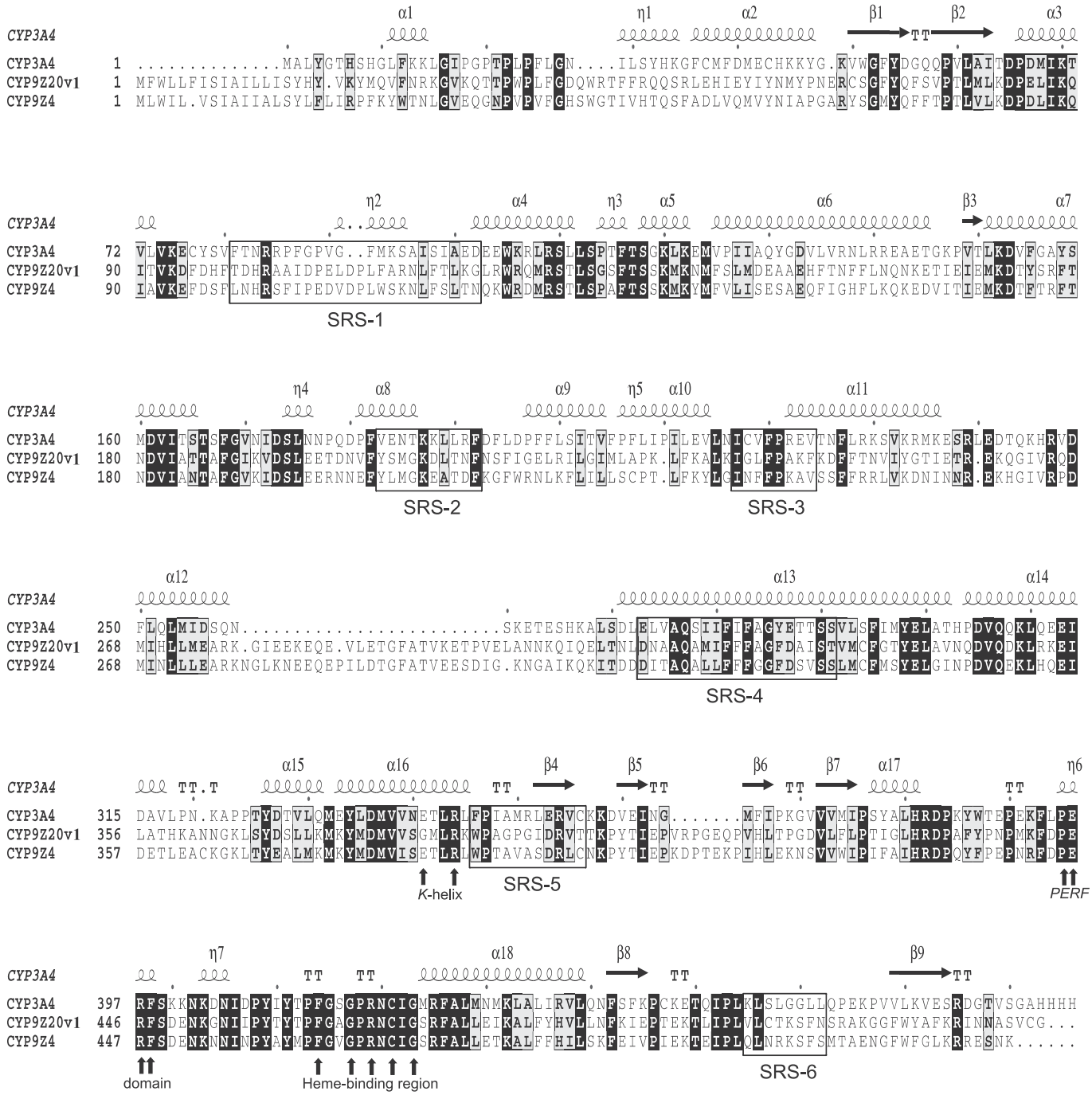


Fig. 4. Multiple sequence alignment and secondary structure elements assignment. The alignment included *CYP9Z20v1* (accession no. HQ113142) from *D. rhizophagus*, the predicted *CYP9Z4* from *T. castaneum* (accession no. NP_001164248) and the mammalian CYP3A4 protein sequence. Substrate recognition sites (SRSs) 1–6 were manually determined. The heme-binding region (FXXGXRXCXG), PERF domain (PXRX) and K-helix (EXXR) are indicated with arrows. The alpha helices are marked as alpha or beta based on the automatic assignment according to the template of the CYP3A4 protein structure in the program ESPript.

Mamestra brassicae L, the expression of genes from the CYP4, CYP6 and CYP9 in the antennae has been associated with exposure to xenobiotics and odor molecules and have been proposed to play a role in deactivation of pheromone molecules in the antennae (Maïbèche-Coisne et al., 2002, 2004a, 2005; Wang et al., 1999; Wojtasek and Leal, 1999).

In addition, our results suggest that P450 enzymes in antennae and gut of *D. rhizophagus* perform different functions depending on the identity and chirality of encountered monoterpenes. Presumably, this function is the oxidation of the specific monoterpene or enantiomer, as studies have demonstrated that bark beetle P450 enzymes can hydrolyze monoterpenes. For example, Sandstrom et al. (2006) showed that the P450 enzyme encoded by the *CYP9T2* gene and its orthologous *CYP9T1* gene in the midgut of *I. pini* and *I. confusus*, respectively, hydroxylate myrcene to an intermediate enantiomeric blend of 80% (−): 20% (+) ipsdienol, which is catalyzed by the ipsdienol dehydrogenase enzyme to ipsdienone (Figueroa-Teran et al., 2012). The (−) enantiomer of ipsdienol functions as aggregation pheromone in the *Ips* spp.

The theoretical analysis that was performed to infer the cellular localization of the deduced cytochrome P450 enzymes in the antennae

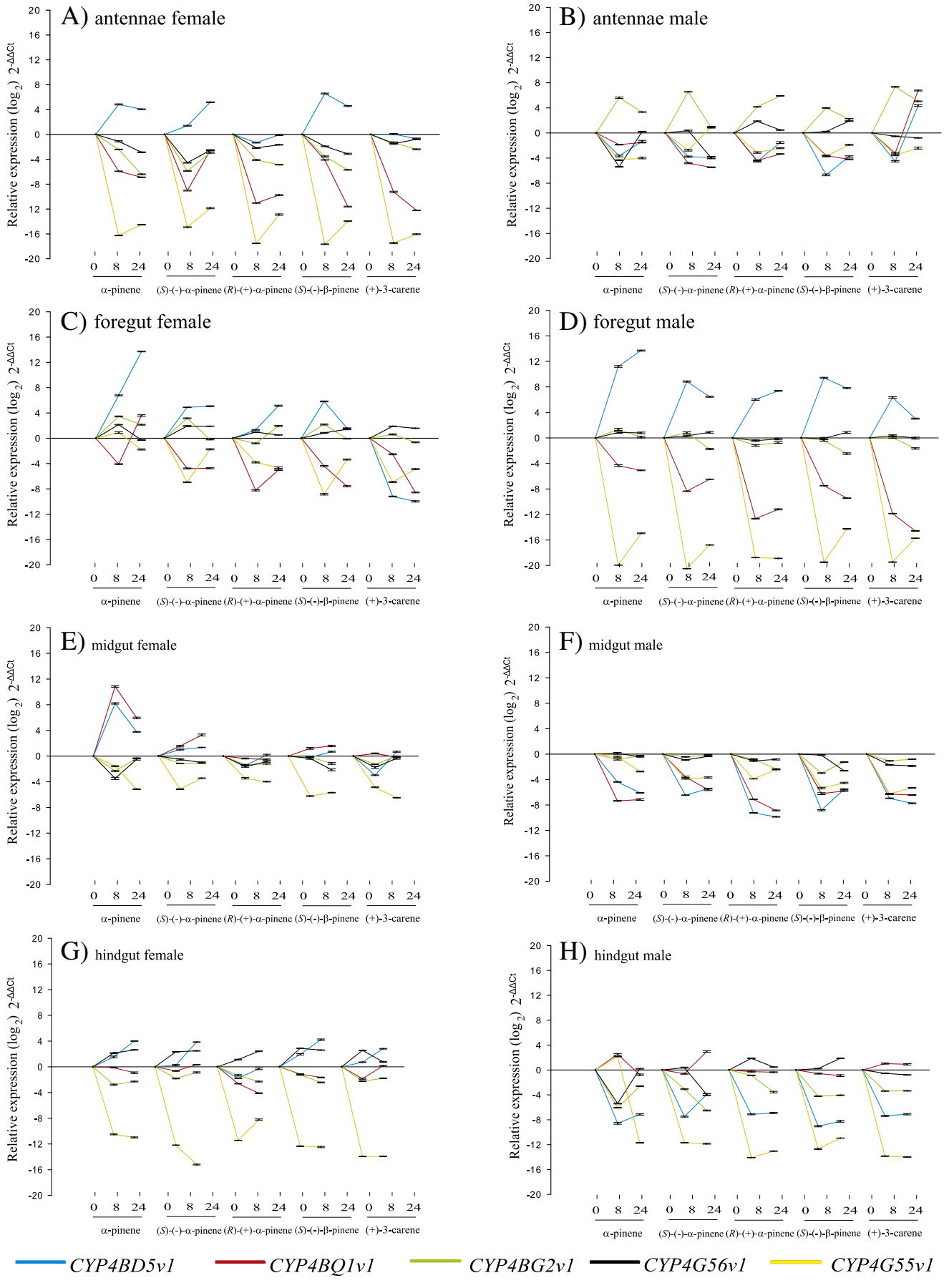

A) antennae female
B) antennae male
C) foregut female
D) foregut male
E) midgut female
F) midgut male
G) hindgut female
H) hindgut male
Relative expression (log2) 2-ΔΔCt
20 16 12 8 4 0 -4 -8 -12 -16 -20
0 8 24
α-pinene
(S)-(-)-α-pinene
(R)-(+)-α-pinene
(S)-(-)-β-pinene
(+)-3-carene
CYP4BD5v1
CYP4BQ1v1
CYP4BG2v1
CYP4G56v1
CYP4G55v1

Table 2
Three-way ANOVA results of each *CYP* gene in *Dendroctonus rhizophagus* females.

Gene/Interaction	df	*CYP4BD5v1*	*CYP4BQ1v1*	*CYP4BG2v1*	*CYP4G56v1*	*CYP6BW5v1*	*CYP6DJ1v1*	*CYP6DJ2v1*	*CYP9Z20v1*
Region (R)	3	1039.2	31,558.5	10,155.9	8076.0	9818.6	6707.3	2216.9	69,917.5
Stimuli (S)	5	19,040.3	7488.6	1095.9	53.21	2084.1	1650.1	686.0	4438.3
Time (T)	2	10,926.4	13,285.2	3929.5	0.240*	10,285.4	7118.9	139.3	11,988.4
R∗S	15	5108.8	1970.7	854.7	480.5	628.0	794.3	805.9	5342.3
R∗T	6	668.8	7973.6	2799.4	2089.0	3026.3	2029.9	932.9	17,737.7
S∗T	10	5271.2	2784.0	500.8	76.8	650.4	881.2	810.0	1262.4
R∗S∗T	30	2088.1	1369.5	571.1	285.8	402.3	362.8	643.7	1488.5

All *F* values were statistically significant at $p<0.001$, except value with *.

Table 3
Three-way ANOVA results of each *CYP* gene in *Dendroctonus rhizophagus* males.

Gene/interaction	*df*	*CYP4BD5v1*	*CYP4BQ1v1*	*CYP4BG2v1*	*CYP4G56v1*	*CYP6BW5v1*	*CYP6DJ1v1*	*CYP6DJ2v1*	*CYP9Z20v1*
Region (R)	3	1002.0	31,647.5	15,582.5	387.7	3468.5	1169.2	59,562.8	4183.7
Stimuli (S)	5	307.6	7156.4	350.5	474.7	3204.0	98.7	1555.5	668.4
Time (T)	2	1788.4	35,346.0	927.3	249.4	18,168.3	32.7	4072.5	2101.5
R∗S	15	62.7	3244.9	820.6	370.9	375.6	73.2	4221.3	277.9
R∗T	6	297.8	8409.9	4153.2	127.4	1039.7	326.6	15,170.5	1312.5
S∗T	10	97.0	2080.3	365.8	624.6	976.8	31.4	2146.8	218.0
R∗S∗T	30	27.4	1419.8	403.4	364.4	187.3	32.1	2285.7	167.0

All *F* values were significant at $P<0.001$.

and gut from *D. rhizophagus* indicates that they are probably anchored to the outer face of the endoplasmic reticulum. If this inference is true for antenna-localized cytochrome P450 enzymes, then monoterpenes should be actively transported into support cells embedding sensory neurons probably by odorant binding proteins (OBPs), as assumed for other intracellular odor degrading enzymes (ODEs), such as GST and dehydrogenase reductase (SDR) (Wang et al., 1999). However, because other odorant compounds or pheromones could rapidly be degraded by ODEs (e.g. esterases and aldehydes-oxidases) within extracellular sensilla lumen (Maïbèche-Coisne et al., 2004b; Vogt, 2003), the role of P450 enzymes in the specific monoterpenes degrading in the olfactory process should be confirmed in future studies.

Because monoterpenes are volatile and, when they are exuded by damaged phloem, can coat the surface of mining beetles, these compounds may enter the bodies of bark beetles via the respiratory system and the cuticle as well as through the digestive tract (Prates et al., 1998). Thus one might expect that distinct cytochrome P450 genes were differentially expressed in the different organs or anatomical systems of bark beetles. The induction of distinct CYP genes in the *D. rhizophagus* gut is consistent with this idea, and their differential induction seems to occur as a response to the presence of different monoterpenes.

The genes' number expressed in the gut with monoterpenes assayed, and the high cellular diversity and secretory richness observed in their regions in unfed insects of *Dendroctonus* spp. (Díaz et al., 1998; Silva-Olivares et al., 2003) suggest, that the CYP genes have distinct physiological roles in the different regions of this system, related with digestive process, pheromone synthesis and detoxification process. In particular, the presence of a detoxification process that takes place in the midgut is indirectly supported by the cellular damage that monoterpenes and their intermediaries produce in the midgut epithelial cells of these bark beetles (López et al., 2011; Silva-Olivares et al., 2003), the increase in the number of secretory vesicles carrying electron-dense material toward the gut lumen (Hall et al., 2002a,b; Nardi et al., 2002; Silva-Olivares et al., 2003) and the differential induction of CYP genes observed in the midgut, foregut and hindgut in each sex in this study.

The expression patterns observed in the CYP4, CYP6 and CYP9 genes in the antennae and gut regions from *D. rhizophagus* suggest that each CYP genes analyzed is differently induced in each anatomy region, different times in response to the same monoterpenes (Figs. 5–7). The profile of the relative expression of CYP4 genes with respect to its basal expression (t0) level suggests that only *CYP4G55v1* gene is constitutively expressed in all regions, because the rest of the genes were induced by any of the monoterpenes assayed. In the antennae apparently there is a selective and sex-specific expression of these genes, because *CYP4BD5v1* gene was over-expressed in the females with three stimuli, whereas in males the *CYP4BG2v1* gene was over-expressed with all stimuli. In the gut regions the CYP4 gene expression patterns were differential between the regions for each sex, with the exception of the *CYP4BD5v1* gene that showed an over-expression in the foregut. In the midgut and hindgut the expression of all CYP4 genes was low or inhibited. Expression patterns similar to those in unfed *D. rhizophagus* were observed in the majority of the CYP4 genes that were identified in fed *I. paraconfusus* using quantitative real-time PCR (Huber et al., 2007). These profiles of the CYP4 gene expression patterns in both species suggest that the primary biochemical function of CYP4 genes in bark beetles may not be the degradation or transformation of exogenous monoterpenes. Evidence from other insect systems suggests that CYP4 genes are involved in fatty acid hydroxylation (Feyereisen, 2005), biosynthesis and metabolism of pheromones (Blomquist et al., 1994; Sutherland et al., 1998; Tillman-Wall et al., 1992; Warren et al., 2002), including juvenile hormone and those related to the gonadotropic cycle (Davies et al., 2006).

On the other hand, the different expression patterns of the CYP6 and CYP9 genes, with respect to those CYP4 genes (Figs. 6 and 7), suggest

Fig. 5. Quantitative expression of *CYP4* genes (mean ± SE) in antennae and gut after stimulating with vapors of racemic α-pinene, (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene, (*S*)-(−)-β-pinene and (+)-3-carene in *D. rhizophagus* at times 0, 8 and 24 h. Note the logarithmic y-axis. CYP expression was normalized with respect to *CYP4G55v1* gene. Statistically significant differences in the genes expression are indicated in the Tables 2 and 3. $2^{-\Delta\Delta Ct}$ and SE values were transformed at $\log_2$ for their graphing.

Table 4
Tukey test results among region, stimuli and time of the *CYP* gene expression identified in *Dendroctonus rhizophagus*. *CYP4G55v1* and *CYP6DG1v1* genes were jointly analyzed for both sexes.

		Females															
		Antennae				Foregut				Midgut				Hindgut			
CYP4BD5v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	***A***	***a***	***A***	***a***	***A***	***b***	***A***	***b***	***A***	***c***	***A***	***c***	***A***	***d***	***A***	***a***
(*S*)-(-)-α-pinene	t0	***B***	***a***	***B***	***a***	***B***	***b***	***B***	***a***	***B***	***c***	***B***	***b***	***B***	***c***	***A***	***c***
(*R*)-(+)-α-pinene	t0	C	a	***C****	***a***	***C***	***b***	***B***	***b***	C	c	***C****	***c***	C	d	B	d
(*S*)-(-)-β-pinene	t0	***D***	***a***	***D***	***a***	***D***	***b***	***C***	***b***	D	c	***D***	***c***	***D***	***d***	***C***	***d***
(+)-3-carene	t0	***E****	***a***	E	a	E	b	D	b	E	c	***D***	***c***	***E***	***d***	***D***	***d***
CYP4BQ1v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A	a	A	b	***A***	***b***	***A***	***c***	***A***	***c***	A*	d	A	d
(*S*)-(-)-α-pinene	t0	B	a	B	a	B	b	B	b	***B***	***c***	***B***	***c***	B	d	B	d
(*R*)-(+)-α-pinene	t0	C	a	C	a	C	b	C	b	C	c	C	c	C	d	C	d
(*S*)-(-)-β-pinene	t0	D	a	D	a	D	b	D	b	***D***	***c***	***D***	***c***	D	d	D	d
(+)-3-carene	t0	B	a	E	a	E	b	E	b	***E***	***c***	***E****	***c***	E	d	B*	d
CYP4BG2v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A	a	***A***	***b***	***A***	***b***	A	a	A	c	A*	c	A	d
(*S*)-(-)-α-pinene	t0	B	a	B	a	***B***	***b***	B*	b	B	c	B	c	B	d	B	d
(*R*)-(+)-α-pinene	t0	C	a	C	a	C	b	***C***	***b***	C	c	B	c	C	d	C	d
(*S*)-(-)-β-pinene	t0	D	a	D	a	***D***	***b***	B*	b	D*	c	B	c	D	d	D	d
(+)-3-carene	t0	E	a	E	a	***E***	***b***	D	b	B,	a	C*	c	E	c	B*	c
CYP4G56v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A	a	***A***	***b***	A	b	A	c	A	c	***A***	***b***	***A***	***d***
(*S*)-(-)-α-pinene	t0	B	a	B	a	***A***	***b***	***B***	***b***	B	c	B	c	***A***	***d***	***A***	***d***
(*R*)-(+)-α-pinene	t0	C	a	C	a	***B***	***b***	***C***	***b***	C	c	B	c	***B***	***b***	***A***	***d***
(*S*)-(-)-β-pinene	t0	D	a	D	a	***B***	***b***	***D***	***b***	B	c	C	c	***C***	***d***	***A***	***d***
(+)-3-carene	t0	E	a	E	a	***A***	***b***	***D***	***b***	C	c	A	c	***D***	***d***	***B***	***d***

		Males															
		Antennae				Foregut				Midgut				Hindgut			
CYP4BD5v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A*	a	***A***	***b***	***A***	***b***	A*	c	A	a	A	d	A	c
(*S*)-(-)-α-pinene	t0	A	a	B	a	***B***	***b***	***B***	***b***	B	a	A	a	B	c	B	c
(*R*)-(+)-α-pinene	t0	A	a	A*	a	***C***	***b***	***C***	***b***	C	a	B	a	B	c	A	c
(*S*)-(-)-β-pinene	t0	B	a	B	a	***B***	***b***	***C***	***b***	C	a	A	a	A	c	A	c
(+)-3-carene	t0	A	a	***C***	***a***	***C***	***b***	***D***	***b***	B	a	C	c	B	c	A	b
CYP4BQ1v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A	a	A	b	A	b	A	c	A	c	***A***	***d***	A	d
(*S*)-(-)-α-pinene	t0	B	a	B	a	B	b	B	b	B	c	B	a	B	d	***B***	***c***
(*R*)-(+)-α-pinene	t0	C	a	C	a	C	b	C	b	C	c	C	c	C	d	C	d
(*S*)-(-)-β-pinene	t0	D	a	D	a	D	b	D	b	D	c	B	c	B	d	A	d
(+)-3-carene	t0	E	a	***E***	***a***	E	b	E	b	D	c	D	c	***D***	***d***	***D***	***d***
CYP4BG2v1		8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	***A***	***a***	***A***	***a***	***A***	***b***	A*	b	A	c	A	c	A	d	A	d
(*S*)-(-)-α-pinene	t0	***B***	***a***	***B***	***a***	***B***	***b***	B	b	B	c	A*	c	B	d	B	d
(*R*)-(+)-α-pinene	t0	***C***	***a***	***C***	***a***	C	b	C	b	A	c	B	c	C	c	C	d
(*S*)-(-)-β-pinene	t0	***C***	***a***	***D***	***a***	D	b	D	b	B	c	C	c	D	d	D	d
(+)-3-carene	t0	***D***	***a***	***E***	***a***	E*	b	B	b	A	c	D	c	E	d	C	d
CYP4G56v1		8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	***A****	***a***	***A***	***b***	***A***	***b***	A*	c	A	c	A	a	***A****	***a***
(*S*)-(-)-α-pinene	t0	***B***	***a***	B	a	***B***	***a***	***A***	***b***	B	b	A	c	***B***	***a***	B	a
(*R*)-(+)-α-pinene	t0	***C***	***a***	***A***	***a***	C	b	B*	b	B	c	B	c	***C***	***a***	***A***	***a***
(*S*)-(-)-β-pinene	t0	***B****	***a***	***C***	***a***	D*	b	***A***	***b***	C*	b	C	c	***B****	***a***	***C***	***a***
(+)-3-carene	t0	D	a	D	a	***B***	***b***	B*	b	C	c	D	c	D	a	D	a

Table 4 (*continued*)

		Females															
		Antennae				Foregut				Midgut				Hindgut			
CYP6BW5v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	***A***	***a***	A*	a	***A***	***b***	***A***	***b***	***A***	***c***	***A***	***c***	A	d	A	d
(*S*)-(-)-α-pinene	t0	***B***	***a***	***B***	***a***	***B***	***b***	***B***	***b***	***A***	***c***	***B***	***c***	A	d	***B***	***d***
(*R*)-(+)-α-pinene	t0	***C***	***a***	***C***	***a***	***C***	***b***	***C***	***b***	***B***	***c***	***C***	***b***	B	d	C	c
(*S*)-(-)-β-pinene	t0	***D***	***a***	D*	a	***D***	***b***	***D***	***b***	***C***	***c***	***A***	***c***	C	d	D	d
(+)-3-carene	t0	***E***	***a***	E	a	***E***	***b***	***A***	***b***	***D***	***b***	***B***	***c***	A	c	***E***	***d***
CYP6DJ1v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A	a	***A***	***b***	***A***	***b***	A	c	***A***	***c***	A	d	A	d
(*S*)-(-)-α-pinene	t0	B	a	B	a	B	b	B	b	B	b	B	b	B	c	B	b
(*R*)-(+)-α-pinene	t0	A	a	B	a	C	a	C	b	B	b	C	c	B	c	C	d
(*S*)-(-)-β-pinene	t0	C	a	C	a	***D***	***b***	D	b	***C***	***c***	C	c	C	d	D	d
(+)-3-carene	t0	D	a	B	a	E	a	E	b	D	b	B	c	D	c	D	d
CYP6DJ2v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A	a	***A***	***b***	***A***	***b***	A	c	***A***	***c***	***A***	***d***	***A***	***d***
(*S*)-(-)-α-pinene	t0	***B***	***a***	***B***	***a***	***B***	***b***	B*	b	A	c	B	c	***B***	***d***	***A***	***d***
(*R*)-(+)-α-pinene	t0	C	a	C	a	C	b	***C***	***b***	B	b	C	c	C	c	***B***	***b***
(*S*)-(-)-β-pinene	t0	D	a	D	a	***A***	***b***	D	b	C	c	***A***	***c***	***D***	***d***	C	d
(+)-3-carene	t0	E*	a	D	a	D	b	E	b	B	b	C	c	***E***	***c***	***D***	***d***
CYP9Z20v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A	a	***A***	***b***	***A***	***b***	***A***	***c***	***A***	***c***	***A***	***c***	***A***	***d***
(*S*)-(-)-α-pinene	t0	A	a	B	a	***B***	***b***	***B***	***b***	***B***	***c***	***B***	***c***	***B***	***c***	***B***	***c***
(*R*)-(+)-α-pinene	t0	A	a	C	a	***C***	***b***	***C***	***b***	C	c	C	c	C	c	C	c
(*S*)-(-)-β-pinene	t0	B	a	D	a	***D***	***b***	***D***	***b***	***A***	***c***	***D***	***c***	***B***	***d***	D*	d
(+)-3-carene	t0	C	a	D	a	***E***	***b***	***E***	***b***	***D***	***c***	***B***	***c***	D	d	***E***	***d***

		Males															
		Antennae				Foregut				Midgut				Hindgut			
CYP6BW5v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	***A***	***a***	***A***	***a***	***A***	***a***	***A***	***b***	***A***	***b***	***A***	***c***	***A***	***c***	***A***	***d***
(*S*)-(-)-α-pinene	t0	***B***	***a***	***B***	***a***	***B***	***b***	***B***	***b***	***B***	***c***	***B***	***c***	***B***	***d***	***B***	***b***
(*R*)-(+)-α-pinene	t0	***B***	***a***	***C***	***a***	***C***	***b***	***C***	***b***	***C***	***c***	***C***	***c***	***C***	***a***	***A***	***d***
(*S*)-(-)-β-pinene	t0	***B***	***a***	***D***	***a***	***D***	***b***	***D***	***b***	***A***	***c***	***D***	***c***	***D***	***d***	***C***	***d***
(+)-3-carene	t0	***C***	***a***	***C***	***a***	***A***	***b***	***E***	***b***	***B***	***c***	***A***	***c***	***C***	***d***	***A***	***d***
CYP6DJ1v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	***A***	***a***	A*	a	A	a	***A***	***b***	***A***	***b***	A	c	***A***	***c***
(*S*)-(-)-α-pinene	t0	A	a	B	a	B	b	B	b	***B***	***c***	***B***	***c***	A	d	B	d
(*R*)-(+)-α-pinene	t0	B	a	C	a	C	a	C	a	***C***	***b***	***A***	***b***	B	b	A	c
(*S*)-(-)-β-pinene	t0	B	a	D	a	C	a	A	b	D *	b	***A***	***c***	C*	b	C*	d
(+)-3-carene	t0	C	a	C	a	C	b	C	a	***C***	***b***	***C****	***b***	C	b	A	c
CYP6DJ2v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	***A***	***a***	***A***	***a***	***A***	***b***	A	b	***A***	***c***	A	c	***A***	***d***	***A***	***d***
(*S*)-(-)-α-pinene	t0	***B***	***a***	***B***	***a***	B	b	***B***	***b***	B	c	***B***	***c***	***B***	***d***	***B***	***d***
(*R*)-(+)-α-pinene	t0	***C***	***a***	***C***	***a***	B	b	A	b	C	c	C	c	***C***	***d***	***C***	***d***
(*S*)-(-)-β-pinene	t0	***D***	***a***	***D***	***a***	C	b	***C***	***b***	D	c	D	c	***D***	***d***	***D***	***d***
(+)-3-carene	t0	***E***	***a***	***E***	***a***	D	b	D	b	***E***	***c***	A	c	***E***	***d***	***E***	***d***
CYP9Z20v1	0 h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	***A***	***a***	***A***	***a***	***A***	***b***	***A***	***b***	***A****	***c***	***A***	***c***	***A***	***c***	***A***	***d***
(*S*)-(-)-α-pinene	t0	B	a	B	a	***B***	***b***	***B***	***b***	***B***	***c***	***B***	***b***	***B****	***d***	***B***	***c***
(*R*)-(+)-α-pinene	t0	C	a	C	a	***C***	***b***	***C***	***b***	***B***	***c***	C*	c	C	c	C	d
(*S*)-(-)-β-pinene	t0	B	a	B	a	***D***	***b***	***D***	***b***	***C***	***c***	***A***	***c***	***D***	***c***	***D***	***a***
(+)-3-carene	t0	B	a	D*	a	***B***	***b***	***E***	***b***	***D***	***c***	***A***	***b***	E	d	***E***	***c***

(*continued on next page*)

Table 4 (*continued*)

		Both sexes															
		Antennae				Foregut				Midgut				Hindgut			
CYP4g55v1	0h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A	a	***A***	***b***	A	a	A*	b	A	b	A	a	A	a
(*S*)-(-)-α-pinene	t0	A	a	B	a	B	b	A	b	B	b	A*	b	B	c	A	c
(*R*)-(+)-α-pinene	t0	A	a	A	a	C	b	A	b	C	b	A*	b	B	c	A	c
(*S*)-(-)-β-pinene	t0	A	a	C	a	C	b	A	b	B	c	A	c	B	d	A	d
(+)-3-carene	t0	A	a	A	a	B	b	A	b	B	b	A	b	B	c	A	c
CYP6DG1v1	0h	8 h		24 h		8 h		24 h		8 h		24 h		8 h		24 h	
α-pinene	t0	A	a	A*	a	***A***	***b***	***A***	***b***	A	a	***A***	***a***	***A***	***b***	***A***	***b***
(*S*)-(-)-α-pinene	t0	A	a	B	a	***B***	***b***	B	b	***B****	***c***	***A***	***c***	***B***	***b***	***A***	***d***
(*R*)-(+)-α-pinene	t0	A	a	C*	a	***C****	***b***	***A***	***b***	***B****	***b***	***B****	***c***	***B***	***d***	***B***	***b***
(*S*)-(-)-β-pinene	t0	B	a	A	a	***B***	***b***	***C***	***b***	***B****	***c***	***B***	***c***	***B***	***b***	***A***	***b***
(+)-3-carene	t0	***C***	***a***	D*	a	***A***	***b***	***A***	***b***	***B****	***a***	***A***	***b***	***B***	***b***	***C***	***c***

The letters in the white or blue columns show results of pairwise multiple comparisons among stimuli within each anatomical region at 8 h and 24 h, respectively. The letters in the gray and light red columns show results of pairwise multiple comparisons among anatomical region by each stimulus at 8 h and 24 h, respectively. The same letter within each columns and each rows indicates statistically no significant differences; * = no significant differences in the expression gene with respect to zero time. Italic letters in bold indicate where the gene was expressed. Underlined letters indicate where the gene was expressed in the males in the *CYP4G55v1* and *CYP6DG1v1* genes.

that these families play a role in the monoterpene metabolism in *D. rhizophagus* antennae and gut. Likewise to antenna CYP4 genes, the expression of CYP6 genes in these structures apparently is sex-specific, because males expressed different genes (*CYP6DJ2v1*♂, *CYP6DG1v1* ♂) with the same stimuli, with the exception of *CYP6BW5v1* gene that was expressed by both sexes.

The expression profile of these genes in the *D. rhizophagus* gut suggests that the cytochrome P450 enzyme encoded by these genes might be involved in the detoxification process, because they were over-expressed with almost all stimuli and any time. The CYP6 family is unique to the class Insecta and its biochemical function has essentially been associated with the metabolism of plant chemicals in phytophagous insects (David et al., 2006; see Schuler, 2011 and citation therein); however, little is known about its function in bark beetles. Thus, it is necessary to carry out studies of heterologous expression with the P450 protein to evaluate whether these genes are involved in the hydroxylation of monoterpenes in bark beetles. In addition, other authors have reported that CYP6 genes could participate in the epoxidation of fatty acid in bark beetles. For example, Aw et al. (2010) suggest that a cluster of three genes in *D. ponderosae* males (cytochrome P450 (CYP6CR1), a putative dehydrogenase and an unknown protein) is involved in terminal steps of the *exo*-brevicomin biosynthesis (a pheromone produced by males). Particularly, the cytochrome P450 is hypothesized to catalyze the epoxidation of fatty acyl-derived precursor, (Z)-6-nonen-2-one to 6,7-epoxynonan-2-one, a precursor of this pheromone (Vanderwell et al., 1992).

The expression profile of *CYP9Z20v1* was not sex-specific, however it was only over-expressed in the female foregut with all times and stimuli, except with α-pinene racemic mixture. This result suggests that this gene could have an important role in the detoxification process. Others studies have been reported that the cytochrome P450 enzymes coded by *CYP9T1* and *CYPT92* genes from *I. confusus* and *I. pini* bark beetles can hydroxylate myrcene to ipsdienol, a component of the aggregation pheromone of these species (Sandstrom et al., 2006, 2008).

While there is increasing evidence that cytochrome P450 enzymes are involved in the *de novo* synthesis of hemiterpenoid and monoterpenoid pheromones via the mevalonate pathway or the cyclization of monounsaturated ketones (Seybold et al., 1995), our results are consistent with the hypothesis that CYP genes are involved in the generalized metabolic process triggered in bark beetles in response to host-produced toxins. Enhanced knowledge of the role of cytochrome P450 enzymes in the detoxification of exogenous compounds and pheromone synthesis is necessary to furthering understanding of the relationship between bark beetles and their host trees.

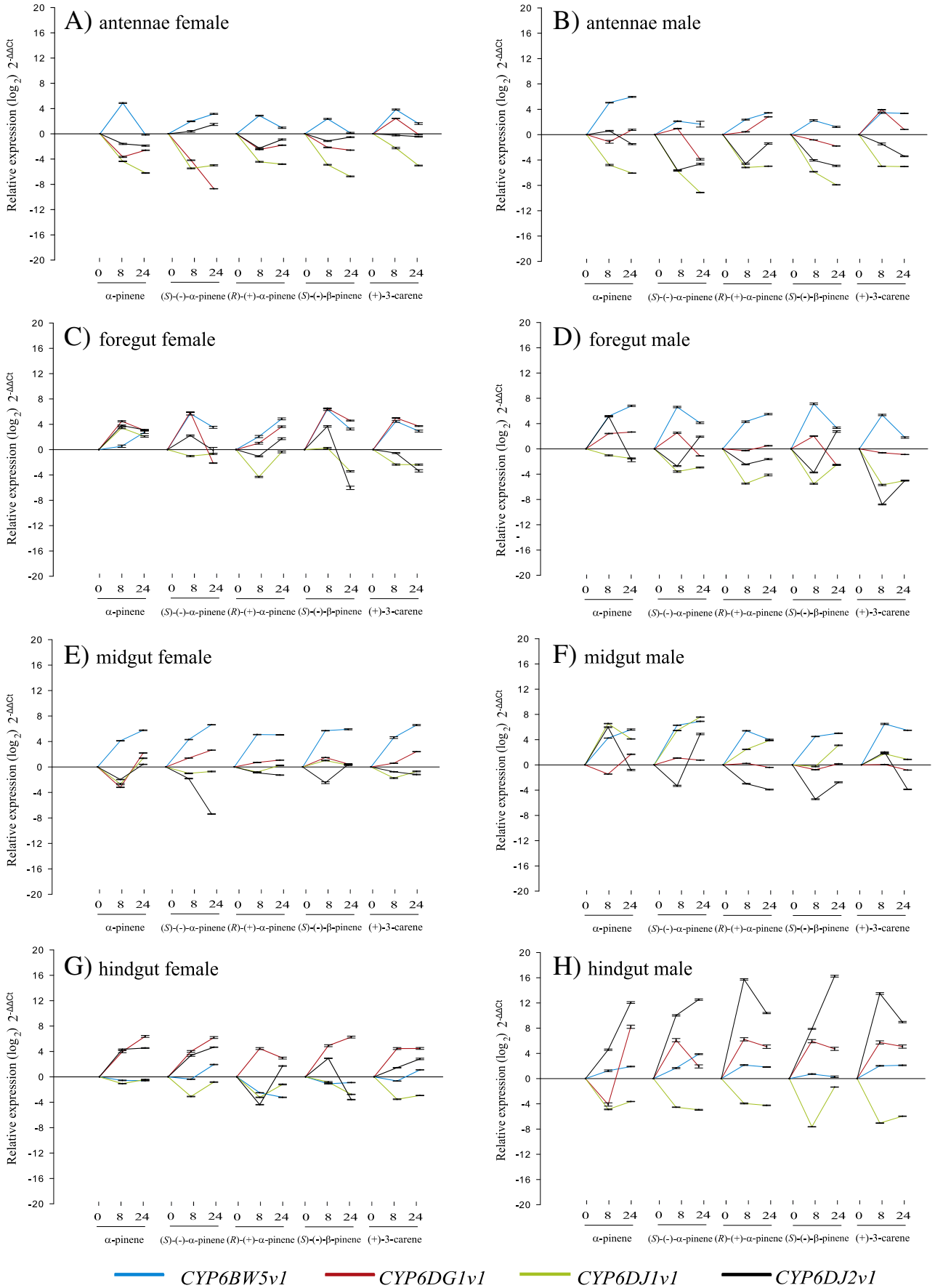
5. Conclusion

Ten new CYP genes were identified in the antenna and gut from *D. rhizophagus* after their exposure to the main monoterpenes of their host tree. These P450s belong to two clans (CYP3 and CYP4), three families (CYP4, CYP6 and CYP9) and five subfamilies. Almost all the genes showed statistically significant differences in their expression level between sexes. A differential behavior of these genes was found in the females and males from *D. rhizophagus* under different stimuli, time and anatomical regions. The expression profile of these genes in the antenna was selective and sex-specific. The expression patterns in the gut region of each CYP genes suggest that they are differently induced in each of its regions and time in response to the same monoterpenes. These expression profiles suggest that some of these genes, particularly CYP6 family, could be involved in the detoxification process during the colonization phase of the host trees.

Acknowledgments

We are grateful to Jesús I. Morales Jiménez and Gabriel Obregon, for their assistance in collecting insects, and to administrator Jaime Chávez and COPAMEX for providing us access to installations and for his logistic support in San Juanito Chihuahua. We thank Graciela Castro Escarpulli, Javier Victor, Isabel Salazar, Jorge Macías Sámano, and Gloria León Avila for their comments and valuable suggestions regarding the manuscript. The project was funded by the Comisión Nacional Forestal-Consejo Nacional de Ciencia y Tecnología (CONAFOR-CONACYT, 69539), Consejo Nacional de Ciencia y Tecnología (181337/CB2012) and Secretaría de Investigación y Posgrado-IPN (SIP-20090576). This work was part of CCR and MFL Ph Ds. dissertations. They were fellows of Consejo Nacional de Ciencia y Tecnología (202060, 175839 respectively) and Programa Institucional de Formación de Investigadores del Instituto

Fig. 6. Quantitative expression of *CYP6* genes (mean ± SE) in antennae and gut after stimulating with vapors of racemic α-pinene, (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene, (*S*)-(−)-β-pinene and (+)-3-carene in *D. rhizophagus* at times 0, 8 and 24 h. Note the logarithmic y-axis. CYP expression was normalized with respect to *CYP4G55v1* gene. Statistically significant differences in the genes expression are indicated in the Tables 2 and 3. $2^{-\Delta\Delta Ct}$ and SE values were transformed at $\log_2$ for their graphing.


A) antennae female
B) antennae male
C) foregut female
D) foregut male
E) midgut female
F) midgut male
G) hindgut female
H) hindgut male
Relative expression (log2) 2-ΔΔCt
20 16 12 8 4 0 -4 -8 -12 -16 -20
0 8 24
α-pinene
(S)-(-)-α-pinene
(R)-(+)-α-pinene
(S)-(-)-β-pinene
(+)-3-carene
CYP6BW5v1
CYP6DG1v1
CYP6DJ1v1
CYP6DJ2v1

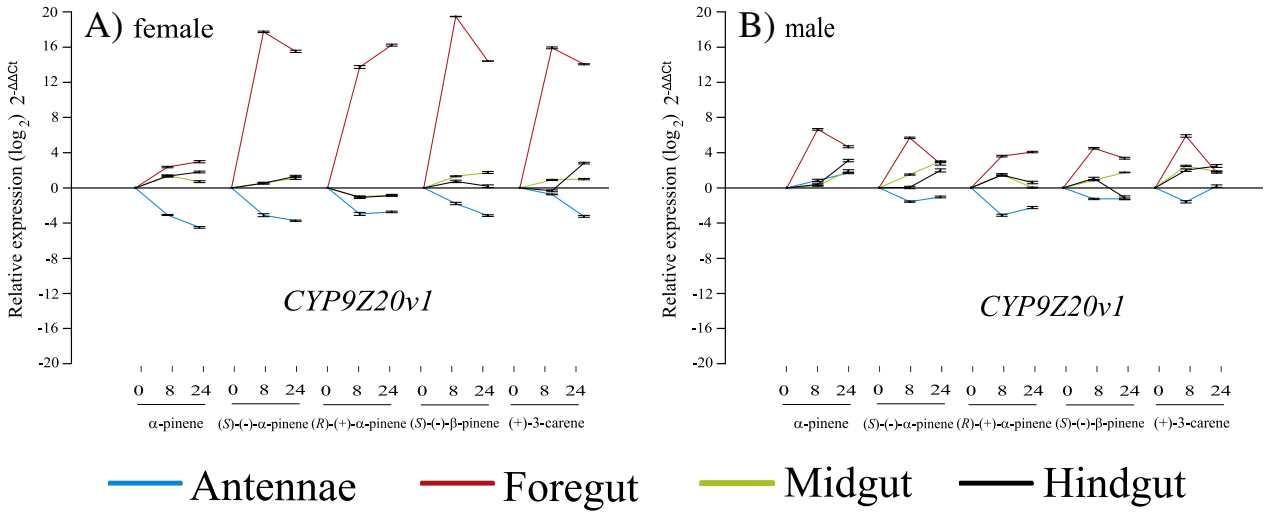


Fig. 7. Quantitative expression of *CYP9* genes (mean ± SE) in antennae and gut after stimulating with vapors of racemic α-pinene, (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene, (*S*)-(−)-β-pinene and (+)-3-carene in *D. rhizophagus* at times 0, 8 and 24 h. Note the logarithmic y-axis. CYP expression was normalized with respect to *CYP4G55v1* gene. Statistically significant differences in the gene expression are indicated in the Tables 2 and 3. $2^{-\Delta\Delta Ct}$ and SE values were transformed at $\log_2$ for their graphing.

Politécnico Nacional (PIFI-IPN). Karina Cesar Ayala and Verónica Pineda Martínez were CONAFOR-CONACYT and PIFI-IPN fellows.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2012.11.059.

References

Abascal, F., Zardoya, R., Posada, D., 2005. ProtTest: selection of best it models of protein evolution. Bioinformatics 21, 2104–2105.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Andersen, C.L., Jensen, J.L., Orntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250.

Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. 55, 539–552.

Aw, T., et al., 2010. Functional genomics of mountain pine beetle (*Dendroctonus ponderosae*) midguts and fat bodies. BMC Genomics 11, 1–12.

Blomquist, G.J., Guo, L., Gu, P., Blomquist, C., Reitz, R.C., Reed, J.R., 1994. Methyl-branched fatty acid and their biosynthesis in the housefly, *Musca domestica* L. (Diptera: Muscidae). Insect Biochem. Mol. Biol. 24, 803–810.

Blomquist, G.J., et al., 2010. Pheromone production in bark beetles. Insect Biochem. Mol. Biol. 40, 699–712.

Byers, J.A., 1995. Host tree chemistry affecting colonization in bark beetles. In: Card, R.R., Bell, W.J. (Eds.), Chemical Ecology of Insects. Academic Press, New York, pp. 154–213.

Campanella, J.J., Bitincka, L., Smalley, J., 2003. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinforma. 4, 29.

Cano-Ramírez, C., Armendáriz-Toledano, F., Macías-Sámano, J.E., Sullivan, B.T., Zúñiga, G., 2012. Electrophysiological and behavioral responses of the bark beetle *Dendroctonus rhizophagus* to volatiles from host pines and conspecifics. J. Chem. Ecol. 38, 512–524.

Christiansen, E., Waring, R.H., Berryman, A.A., 1987. Resistance of conifers to bark beetle attack: searching for general relationships. For. Ecol. Manag. 22, 89–106.

David, J.P., Boyer, S., Mesneau, A., Ball, A., Ranson, H., Dauphin-Villemant, C., 2006. Involvement of cytochrome P450 monooxygenases in the response of mosquito larvae to dietary plant xenobiotics. Insect Biochem. Mol. Biol. 36, 410–420.

Davies, L., Williams, D.R., Turner, P.C., Rees, H.H., 2006. Characterization in relation to development of an ecdysteroid agonist-responsive cytochrome P450, *CYP18A1*, in Lepidoptera. Arch. Biochem. Biophys. 453, 4–12.

Díaz, E., Cisneros, R., Zúñiga, G., Uria-Galicia, E., 1998. Comparative anatomical and histological study of the alimentary canal of *Dendroctonus parallelocollis*, *D. rhizophagus* and *D. valens* (Coleoptera: Scolytidae). Ann. Entomol. Soc. Am. 91, 479–487.

Dickens, J.C., Visser, J.H., Van den Pers, J.N.C., 1992. Detection and deactivation of pheromone and plant odor components by the Beet Armyworm, *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). J. Insect Physiol. 39, 503–516.

Dowd, F.D., Smith, C.M., Sparks, T.C., 1983. Detoxification of plant toxins by insects. Insect Biochem. 13, 453–468.

Dunkov, B.C., Rodríguez-Arnaiz, R., Pittendrigh, B., Ffrench-Constant, R.H., Feyereisen, R., 1996. Cytochrome P450 gene clusters in *Drosophyla melanogaster*. Mol. Gen. Genet. 251, 290–297.

Emanuelsson, O., Nielsen, H., Brunak, S., von Heijne, G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300, 1005–1016.

Feyereisen, R., 1999. Insect P450 enzymes. Annu. Rev. Entomol. 44, 507–533.

Feyereisen, R., 2005. Insect cytochrome P450. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science — Biochemistry and Molecular Biology. Elsevier, Oxford, pp. 1–77 (United Kingdom).

Feyereisen, R., 2006. Evolution of insect P450. Biochem. Soc. Trans. 46, 1252–1255.

Figueroa-Teran, R., Welch, W.H., Blomquist, G.J., Tittiger, C., 2012. Ipsdienol dehydrogenase (IDOLDH): a novel oxidoreductase important for *Ips pini* pheromone production. Insect Biochem. Mol. Biol. 42, 81–90.

Franceschi, V.R., Krokene, P., Christiansen, E., Krekling, T., 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytol. 167, 353–376.

Gasteiger, E., et al., 2005. Protein identification and analysis tools on the ExPASy server. In: Walker, J.M. (Ed.), The Proteomics Protocols Handbook. Humana Press, Totowa, New Jersey, pp. 571–607.

Gouet, P., Robert, X., Courcelle, E., 2003. ESPript/ENDscript: extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Res. 31, 3320–3323.

Gouy, M., Guindon, S., Gascuel, O., 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27, 221–224.

Guengerich, F.P., 1990. Enzymatic oxidation of xenobiotic chemical. CRC Crit. Rev. Biochem. Mol. Biol. 25, 97–153.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Hahn, M.W., 2009. Distinguishing among evolutionary models for the maintenance of gene duplicates. J. Hered. 100, 605–617.

Hall, G.M., et al., 2002a. Midgut tissue of male engraver, *Ips pini; synthesizes* monoterpenoid pheromone component ipsdienol *de novo*. Naturwissenschaften 89, 79–83.

Hall, G.M., et al., 2002b. Male Jeffrey pine beetle, *Dendroctonus jeffreyi*, synthesizes the pheromone component frontalin in anterior midgut tissue. Insect Biochem. Mol. Biol. 32, 1525–1532.

Hanover, J.W., 1975. Physiology of tree resistance to insects. Annu. Rev. Entomol. 20, 75–95.

Horton, P., et al., 2007. Wolf PSORT: protein localization predictor. Nucleic Acids Res. 35, 585–587.

Huber, D.P.W., Erickson, M.L., Leutenegger, C.M., Bohlmann, J., Seybold, S.J., 2007. Isolation and extreme sex-specific expression of cytochrome P450 genes in the bark beetle, *Ips paraconfusus*, following feeding on the phloem of host ponderosa pine, *Pinus ponderosa*. Insect Mol. Biol. 16, 335–349.

Hunt, D.W.A., Smirle, M.J., 1988. Partial inhibition of pheromone production in *Dendroctonus ponderosae* (Coleoptera: Scolytidae) by polysubstrate monooxygenase inhibitors. J. Chem. Ecol. 14, 529–536.

Kasai, S., Weerashinghe, I.S., Shono, T., Yamakawa, M., 2000. Molecular cloning, nucleotide sequence and gene expression of a cytochrome P450 (CYP6F1) from the pyrethroid-resistant mosquito, *Culex quinquefasciatus* Say. Insect Biochem. Mol. Biol. 30, 163–171.

Keeling, C., Bohlmann, J., 2006. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytol. 170, 657–675.

Keeling, C.I., et al., 2012. Transcriptome and full-length cDNA resources for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a major insect pest of pine forests. Insect Biochem. Mol. Biol. 42, 525–536.
Le, S.Q., Gascuel, O., 2008. An improved general amino acid replacement matrix. Mol. Biol. Evol. 25, 1307–1320.
Livak, K.J., Schmittgen, T.D., 2008. Analyzing real-time PCR data by the comparative C_T method. Nat. Protoc. 3, 1101–1108.
López, M.F., Cano-Ramírez, C., Shibayama, M., Zúñiga, G., 2011. α-Pinene and myrcene induce ultrastructural change in the midgut of *Dendroctonus valens* (Coleoptera: Curculionida: Scolytinae). Ann. Entomol. Soc. Am. 104, 553–561.
Lyon, R.L., 1958. Useful secondary sex character in *Dendroctonus* Bark Beetles. Can. Entomol. 90, 552–558.
Maïbèche-Coisne, M., Jacquin-Joly, E., Francois, M.C., Le Nagnan-, M.P., 2002. cDNA cloning of biotransformation enzymes belonging to the cytochrome P450 family in the antennae of the noctuid moth *Mamestra brassicae*. Insect Mol. Biol. 11, 273–281.
Maïbèche-Coisne, M., Nikonov, A.A., Ishida, Y., Jacquin-Joly, E., Leal, W., 2004a. Pheromone anosmia in a scarab beetle induced by *in vivo* inhibition of a pheromone-degrading enzyme. PNAS 101, 11459–11464.
Maïbèche-Coisne, M., Merlin, C., Francois, M.C., Queguiner, I., Porcheron, P., Jacquin-Joly, E., 2004b. Putative odorant-degrading esterase cDNA from the moth *Mamestra brassicae*: cloning and expression patterns in male and female antennae. Chem. Senses 28, 381–390.
Maïbèche-Coisne, M., Merlin, C., Francois, M.C., Porcheron, P., Jacquin-Joly, E., 2005. P450 and P450 reductase cDNAs from the moth *Mamestra brassicae*: cloning and expression patterns in male antennae. Gene 346, 195–203.
McKenna, D.D., Sequeira, A.S., Marvaldi, A.E., Farrell, B.D., 2009. Temporal lags and overlap in the diversification of weevils and flowering plants. PNAS 106, 7083–7088.
Mendoza, M.G., Salinas-Moreno, Y., Olivo-Martínez, A., Zúñiga, G., 2011. Factors influencing the geographical distribution of *Dendroctonus rhizophagus* (Coleoptera: Curculionidae: Scolytinae) in the Sierra Madre Occidental, México. Environ. Entomol. 40, 549–559.
Namiky, T., Niwa, R., Sakudoh, T., Shirai, K., Takeuchi, H., Kataoka, H., 2005. Cytochrome P450 CYP307A1/Spook: a regulator for ecdysone synthesis in insects. Biochem. Biophys. Res. Commun. 337, 367–374.
Nardi, J.B., Young, A.G., Ujhelyi, E., Tittiger, C., Lehane, M.J., Blomquist, G.J., 2002. Specialization of midgut cells for synthesis of male isoprenoid pheromone components in two scolytid beetles, *Dendroctonus jeffreyi* and *Ips pini*. Tissue Cell 34, 221–231.
Nelson, D.R., 2011. Progress in tracing the evolutionary paths of cytochrome P450. Biochim. Biophys. Acta 1814, 14–18.
Nelson, D.R., Strobel, H.W., 1987. Evolution of cytochrome P-450 proteins. Mol. Biol. Evol. 4, 572–593.
Nelson, D.R., et al., 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6, 1–41.
Prates, H.T., Santos, J.P., Waquil, J.M., Fabris, J.D., Oliveira, A.B., Foster, J.E., 1998. Insecticidal activity of monoterpenes against *Ryzopertha dominica* (F.) and *Tribolium castaneum* (Herbst). J. Stored Prod. Res. 34, 243–249.
Rongnoparut, P., Boonsuepsakul, S., Chareonviriyaphap, T., Thanomsing, N., 2003. Cloning of cytochrome P450, *CYP6P5*, and *CYP6AA2* from *Anopheles minimus* resistant to deltamethrin. J. Vector Ecol. 28, 150–158.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning, a Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York.
Sánchez-Martínez, G., Wagner, M.R., 2009. Host preference and attack pattern of *Dendroctonus rhizophagus* (Coleoptera: Curculionidae: Scolytinae): a bark beetle specialist on pine regeneration. Environ. Entomol. 38, 1197–1204.
Sandstrom, P., Welch, W.H., Blomquist, G.J., Tittiger, C., 2006. Functional expression of a bark beetle cytochrome P450 that hydroxylates myrcene to ipsdienol. Insect Biochem. Mol. Biol. 36, 835–845.
Sandstrom, P., Ginzel, M.D., Bearfield, J.C., Welch, W.H., Blomquist, G.J., Tittiger, C., 2008. Myrcene hydroxylases do not determine enentiomeric composition of pheromonal ipsdienol in *Ips* spp. J. Chem. Ecol. 34, 1584–1592.
Schuler, M.A., 2011. P450s in plant–insect interactions. Biochim. Biophys. Acta 1814, 36–45.
Seybold, S.J., Quilici, D.R., Tillman, J.A., Vanderwel, D., Wood, D.L., Blomquist, G.J., 1995. De novo biosynthesis of the aggregation pheromone components ipsenol and ipsdienol by the pine bark beetle *Ips paraconfusus* Lanier and *Ips pini* (Say) (Coleoptera: Scolytidae). Proc. Natl. Acad. Sci. U. S. A. 92, 8393–8397.
Seybold, S.J., Huber, D.P.W., Lee, J.C., Graves, A.D., Bohlmann, J., 2006. Pine monoterpenes and pine bark beetles: a marriage of convenience for defense and chemical communication. Phytochem. Rev. 5, 143–178.
Silva-Olivares, A., Díaz, E., Shibayama, M., Tsutsumi, V., Cisneros, R., Zúñiga, G., 2003. Ultrastructural study of the midgut and hindgut in eight species of the genus *Dendroctonus* Erichson (Coleoptera: Scolytidae). Ann. Entomol. Soc. Am. 96, 883–900.
Smith, R.H., 1965. Effect of monoterpene vapors on the western pine beetle. J. Econ. Entomol. 58, 509–510.
Smith, R.H., 2000. Xylem Monoterpenes of Pines: Distribution, Variation, Genetics, Function. Pacific Southwest Research Station, Berkeley CA (General Technical Report PSW-GTR-177).
Snyder, M.J., Scott, J.A., Andersen, J.F., Feyereisen, R., 1996. Sampling P450 diversity by cloning polymerase chain reaction products obtained with degenerate primers. Methods Enzymol. 272, 304–312.
Sutherland, T.D., et al., 1998. A cytochrome P450 terpenoid hydroxylase linked to the suppression of insect juvenile hormone synthesis. Proc. Natl. Acad. Sci. U. S. A. 95, 12884–12889.
Tartar, A., Wheeler, M.M., Zhou, X., Coy, M.R., Boucias, D.G., Scharf, M.E., 2009. Parallel metatranscriptome analyses of host and symbiont gene expression in the gut of the termite *Reticulitermes flavipes*. Biotechnol. Biofuels 2, 1–25.
Thomas, J.B., Bright, D.E., 1970. A new species of *Dendroctonus* (Coleoptera: Scolytidae) from Mexico. Can. Entomol. 102, 479–483.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL X windows interface flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Tillman-Wall, J.A., Vanderwel, D., Kuenzli, M.E., Reitz, R.C., Blomquist, G.J., 1992. Regulation of sex pheromone biosynthesis in the housefly, *Musca domestica*: relative contribution of the elongation and reductive step. Arch. Biochem. Biophys. 299, 92–99.
Vanderwell, D., Ochlschlager, A.C., 1987. Biosynthesis of pheromones and endocrine regulation of pheromone production in Coleoptera. In: Prestwich, G.D., Blomquist, G.J. (Eds.), Pheromone Biochemistry. Academic Press, Orlando, pp. 175–215.
Vanderwell, D., Gries, G., Singh, S.M., Borden, J.M., Oehlschlager, A.C., 1992. (*E*)- and (*Z*)-6-nonen-2one: biosynthetic precursor of *endo*- and *exo*-brevicomin in two bark beetles (Coleoptera: Scolytidae). J. Chem. Ecol. 18, 1389–1404.
Vandesompele, J., et al., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 (research0034.1-0034.11).
Vogt, R.G., 2003. Biochemical diversity of odor detection: OBPs, ODEs and SNMPs. In: Blomquist, G., Vogt, R. (Eds.), Insect Pheromone Biochemistry and Molecular Biology. Academic Press, San Diego, pp. 391–445.
Wang, Q., Hasan, G., Pikielny, C., 1999. Preferential expression of biotransformation enzymes in the olfactory organs of *Drosophila melanogaster*, the antennae. J. Biol. Chem. 274, 10309–10315.
Warren, J.T., et al., 2002. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. A. 99, 11043–11048.
White Jr., R.A., Franklin, R.T., Agosin, M., 1979. Conversion of alpha-pinene oxide by rat liver and the bark beetle *Dendroctonus terebrans* microsomal fractions. Pesticide Biochem. Physiol. 10, 233–242.
White Jr., R.A., Agosin, M., Franklin, R.T., Webb, J.W., 1980. Bark beetles pheromones: evidence for physiological synthesis mechanisms and their ecological implications. Zool. Entomol. 90, 255–274.
Wojtasek, H., Leal, W.S., 1999. Degradation of an alkaloid pheromone from the pale-brown chafer, *Phyllopertha diversa* (Coleoptera: Scarabaeidae), by an insect olfactory cytochrome P450. FEBS Lett. 458, 333–336.
Wood, D.L., 1982. The role of pheromones, kairomones and allomones in the host selection and colonization behaviour of bark beetles. Annu. Rev. Entomol. 27, 411–446.