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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*

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Abstract

We have identified cDNAs and characterized the expression of 13 novel cytochrome P450 genes of potential importance in host colonization and reproduction by the California fivespined ips, *Ips paraconfusus*. Twelve are of the Cyp4 family and one is of the Cyp9 family. Following feeding on host *Pinus ponderosa* phloem, bark beetle transcript levels of several of the Cyp4 genes increased or decreased in males only or in both sexes. In one instance (IparaCyp4A5) transcript accumulated significantly in females, but declined significantly in males. The Cyp9 gene (Cyp9T1) transcript levels in males were > 85 000 × higher at 8 h and > 25 000 × higher at 24 h after feeding compared with nonfed controls. Transcript levels in females were approximately 150 × higher at 24 h compared with nonfed controls. Cyp4G27 transcript was present constitutively regardless of sex or feeding and served as a better housekeeping gene than β-actin or 18S rRNA for the real-time TaqMan polymerase chain reaction analysis. The expression patterns of Cyp4AY1, Cyp4BG1, and, especially, Cyp9T1 in males suggest roles for these genes in male-specific aggregation pheromone production. The differential transcript accumulation patterns of these bark beetle P450s provide insight into ecological interactions of *I. paraconfusus* with its host pines.

Keywords: Coleoptera, Scolytidae, cytochrome P450, pheromone biosynthesis, detoxification, real-time polymerase chain reaction.

Introduction

Bark beetles are endophytic parasites of trees and shrubs and have been widely recognized for their ecological and economic significance in forests (Rudinsky, 1962; Furniss & Carolin, 1977). These subcortical insects spend most of their life cycle feeding in the phloem and outer xylem of their host trees. While this location affords some protection for the adults and their brood from predation and abiotic hazards, the initial attack on a live tree also exposes these insects to large concentrations of host-plant-produced defensive toxins. Like other insects, bark beetles probably overcome these defences partially through enzyme-mediated detoxification of host compounds (Krieger *et al*., 1971; Hodgson, 1985; Rose, 1985; Bernays & Chapman, 1994; Berenbaum, 2002). A complementary strategy for survival among many bark beetles, however, is the phenomenon of mass attack of host trees.

In a mass attack, the first insects to arrive at a tree begin to produce powerful aggregation pheromones (Wood, 1982) that serve to attract other individuals of the same species. As arriving insects land on the tree, they excavate an entrance hole through the outer bark and into the phloem. The combined activity of numerous attacking insects and the rapid growth of symbiotic fungi (Paine *et al*., 1997) that the insects vector into their hosts, acts to quickly weaken the tree. When the host tree defences have been overcome, the aggregated bark beetles reproduce, and they and their offspring utilize host phloem for nutrition.

Throughout the last half of the twentieth century, aggregation pheromones of bark beetles were discovered and

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intensively researched (Wood, 1982; Borden, 1985) with the goal of applying the behaviourally active compounds in forest insect pest management operations (Borden, 1994). The first discovery (Silverstein *et al*., 1966) of an aggregation pheromone in a bark beetle (and in any beetle) was that of the California fivespined ips, *Ips paraconfusus* Lanier. The attractant blend consisted of three monoterpenoid compounds that were extracted and isolated from beetle boring dust and faeces (frass) and later synthesized in the lab to prove identity and activity. The three isoprenoid components of the attractant blend were shown to be (–)-2-methyl-6-methylene-7-octen-4-ol [(–)-ipsenol] (+)-*cis*-4,6,6-trimethylbicyclo-[3.1.1]-hept-3-en-2-ol [(+)-*cis*-verbenol], and (+)-2-methyl-6-methylene-2,7-octadien-4-ol [(+)-ipsdienol]. Since the discovery of the male-produced *I. paraconfusus* aggregation pheromone, the pheromone blends of numerous other bark beetles have been elucidated (Borden, 1985; Seybold *et al*., 2000).

Because conifer-infesting bark beetles are exposed to large quantities of tree-produced monoterpenes (Keeling & Bohlmann, 2006; Seybold *et al*., 2006), and because many of the components of their pheromones are terpenoids, it was thought that the insects primarily sequestered the host compounds, hydroxylated them, and then released them as pheromones (Hughes, 1974; Renwick *et al*., 1976a,b; Hendry *et al*., 1980; Vanderwel, 1994). While this phenomenon does occur in the laboratory, more recent laboratory studies have shown that *Ips* spp. (Seybold *et al*., 1995; Tillman *et al*., 1998, 2004; Hall *et al*., 2002a) and *Dendroctonus* spp. bark beetles (Hall *et al*., 2002b; Barkawi *et al*., 2003) also produce pheromone components *de novo* from metabolic precursors.

A pheromone biosynthetic pathway for *Ips* spp. beetles has been proposed (Seybold & Tittiger, 2003), based largely on observations of the fate of radiolabelled acetate or mevalonolactone (Seybold *et al*., 1995; Tillman *et al*., 1998, 2004); observations of pheromone production following application of an enzyme inhibitor (Ivarsson *et al*., 1993); and observations of expression levels of pathway genes following feeding or application of the sesquiterpenoid insect hormone, juvenile hormone III (JHIII) (Tittiger *et al*., 1999, 2003; Keeling *et al*., 2004, 2006; Tillman *et al*., 2004; Gilg *et al*., 2005; Sandstrom *et al*., 2006). Recently, the conversion of geranyl diphosphate to the monoterpene myrcene by whole body extracts of two populations of fed or JHIII-treated male pine engravers, *Ips pini* (Say), has demonstrated the existence of a terpene synthase-like activity in that insect (Martin *et al*., 2003). The final step in a possible biosynthetic pathway from myrcene to ipsdienol and ipsenol is a simple hydroxylation (Hendry *et al*., 1980; Sandstrom *et al*., 2006). Oxidative hydroxylations are often catalysed by cytochromes P450, a large class of ubiquitous enzymes (Feyereisen, 1999, 2005; Omura, 1999; Berenbaum, 2002), and pheromone biosynthesis is a known function of insect P450s (Ahmad *et al*., 1987; Guo *et al*., 1991; Sandstrom *et al*., 2006). Other functions include detoxification of host-plant chemicals and pesticides (Scott *et al*., 1998; Feyereisen, 1999), as well as degradation of pheromone components in antennae (Maïbèche-Coisne *et al*., 2004) and biosynthesis and degradation of hormones (Sutherland *et al*., 1998).

We report the identification of cDNAs for 13 different P450 genes from *I. paraconfusus* and subsequent highly replicated real-time polymerase chain reaction (PCR) analyses of their expression in phloem-fed and unfed male and female insects. The purpose of our study was to identify specific P450s that are likely important in aspects of pheromone biosynthesis, colonization of host material and detoxification of host compounds, and reproductive physiology in this insect. In addition, because *I. paraconfusus* is an important model organism for the study of bark beetle pheromone biosynthesis and molecular biology (Seybold *et al*., 1995; Tittiger *et al*., 1999; Tillman *et al*., 2004) we have identified, tested, and ranked genes that are candidates for use as housekeeping genes (HKG) for future real-time PCR expression analyses with this and related insects.

Results

Thirteen novel cytochromes p450 from Ips paraconfusus

Following successful degenerate and other homology-based PCR and 5′- and 3′-RACE, we recovered full-length cDNA for eight Cyp4 family genes (*Cyp4AY1*, *Cyp4AY2*, *Cyp4BD1*, *Cyp4BE1*, *Cyp4BF1*, *Cyp4BG1*, *Cyp4BH1* and *Cyp4G27*) and substantial sequence data for four other Cyp4 family genes (*Cyp4BE2*, *Cyp4BJ1*, *IparaCyp4A5* and *IparaCyp4cod1*), and one Cyp9 (*Cyp9T1*) gene from *I. paraconfusus* (Table 1). We also recovered substantial sequence information for a Cyp31 family gene (*Cyp31B1*), which seems to be of nematode origin based on sequence homology with known cytochrome P450 cDNAs. These five latter *I. paraconfusus* cDNAs were determined to be incomplete by length, alignment with known arthropod P450s, lack of an identifiable N-terminal signal peptide in cases in which 5′-end data were missing, and knowledge of the method used to obtain the fragment (in the case of the Cyp9 cDNA). The Cyp9 family gene and all of the Cyp4 family genes show substantial identity to known arthropod P450 genes from the same families, and when 5′-end sequence data were present, TargetP (Emanuelsson *et al*., 2000) indicated that all are likely targeted to the endoplasmic reticulum (Table 1). The Cyp31 family gene shows substantial identity to a known nematode P450 gene (Table 1).

Comparisons of deduced amino acid sequences of *Cyp4AY1*, *Cyp4AY2*, *Cyp4BD1*, *Cyp4BF1*, *Cyp4BG1*, *Cyp4BH1* and *Cyp4AW1* (*Phyllopertha diversa*, GenBank accession no. AY605086) (Fig. 1); *Cyp4BE1* and *Cyp4AB1* (*Solenopsis invicta*, GenBank accession no. AY345970) (Fig. 2); *Cyp4G27*, *Cyp4G16* (*Anopheles gambiae*, GenBank

Table 1. Characteristics of cytochrome P450 cDNAs isolated from *Ips paraconfusus* samples and comparisons with known arthropod and nematode cytochromes P450

			Blastp Matches in GenBank				
Ips paraconfusus P450 Name[a]	Full length (FL) or partial (P) cDNA	Recovered ORF size (bp)	P450 name	Organism	GenBank Accession Number	Identity	Signal Peptide Prediction[b]
Cyp4AY1	FL	1534	*Cyp4AW1*	*Phyllopertha diversa*	AAT38512	37%	SP 0.928, mTP 0.036, other 0.158
			Cyp4C39	*Carcinus maenas*	AAQ93010	35%	
			Cyp4C15	*Orconectes limosus*	AAF09264	33%	
Cyp4AY2	FL	1496	*Cyp4AW1*	*Phyllopertha diversa*	AAT38512	38%	SP 0.935, mTP 0.117, other 0.030
			Cyp4AW2	*Phyllopertha diversa*	AAT38513	37%	
			Cyp4C1	*Blaberus discoidalis*	P29981	36%	
Cyp4BD1	FL	1526	*Cyp4AW1*	*Phyllopertha diversa*	AAT38512	43%	SP 0.972, mTP 0.019, other 0.067
			Cyp4AW2	*Phyllopertha diversa*	AAT38513	40%	
			Cyp4C1	*Blaberus discoidalis*	P29981	38%	
Cyp4BE1	FL	1279	*Cyp4C39*	*Carcinus maenas*	AAQ93010	48%	SP 0.764, mTP 0.046, other 0.391
			Cyp4C1	*Blaberus discoidalis*	P29981	44%	
			Cyp4AB1	*Solenopsis invicta*	AAP97090	39%	
Cyp4BE2	P, 3′-end	1347	*Cyp4C39*	*Carcinus maenas*	AAQ93010	44%	no 5′-end data
			Cyp4C1	*Blaberus discoidalis*	P29981	43%	
			Cyp4C29	*Cherax quadricarinatus*	AAL56662	40%	
Cyp4BF1	FL	1601	*Cyp4C39*	*Carcinus maenas*	AAQ93010	41%	SP 0.956, mTP 0.076, other 0.014
			Cyp4C1	*Blaberus discoidalis*	P29981	41%	
			Cyp4C15	*Orconectes limosus*	AAF09264	38%	
Cyp4BG1	FL	1536	*Cyp4Q7*	*Tribolium castaneum*	AAF70496	47%	SP 0.864, mTP 0.163, other 0.031
			Cyp4Q4	*Tribolium castaneum*	AAF70178	46%	
			Cyp4AB2	*Solenopsis invicta*	AAQ90477	40%	
Cyp4BH1	FL	1529	*Cyp4C15*	*Orconectes limosus*	AAF09264	45%	SP 0.819, mTP 0.313, other 0.021
			Cyp4C39	*Carcinus maenas*	AAQ93010	44%	
			Cyp4AW1	*Phyllopertha diversa*	AAT38512	42%	
Cyp4BJ1	P, 5′-end	1506	*Cyp4C1*	*Blaberus discoidalis*	P29981	38%	SP 0.871, mTP 0.226, other 0.007
			Cyp4C39	*Carcinus maenas*	AAQ93010	38%	
			Cyp4C29	*Cherax quadricarinatus*	AAL56662	37%	
Cyp4G27	FL	1798	*Cyp4G25*	*Antheraea yamamai*	BAD81026	67%	SP 0.842, mTP 0.029, other 0.186
			Cyp4G20	*Mamestra brassicae*	AAR26517	65%	
			Cyp4G19	*Blattella germanica*	AAO20251	61%	
IparaCyp4A5	P, fragment	449	*Cyp4C1*	*Blaberus discoidalis*	P29981	56%	no 5′-end data
			Cyp4C39	*Carcinus maenas*	AAQ93010	53%	
			Cyp4U1	*Coptotermes acinaciformis*	AAC03111	52%	
IparaCyp4cod1	P, 3′-end	624	*Cyp4C39*	*Carcinus maenas*	AAQ93010	51%	no 5′-end data
			Cyp4C15	*Orconectes limosus*	AAF09264	49%	
			Cyp4C29	*Cherax quadricarinatus*	AAL56662	44%	
Cyp9T1	P, 3′-end	1539	*Cyp9T2*	*Ips pini*	ABG74909	94%	no 5′-end data
			Cyp9A8	*Spodoptera litura*	AAP80766	38%	
			Cyp9A17	*Helicoverpa armigera*	AAY21809	37%	
Cyp31B1	P, 3′-end	1407	*Cyp31A2*	*Caenorhabditis elegans*	Z68336	54%	no 5′-end data

[a] When sufficient sequence data were available (all but IparaCyp4A5 and IparaCyp4cod1) the formal nomenclature was provided by D. Nelson (Department of Molecular Sciences, University of Tennessee, personal communication).
[b] As predicted by TargetP (Emanuelsson *et al.*, 2000).

accession no. AAL58550, full sequence at http://p450.antibes.inra.fr/genes/11.html), and *Cyp4G25* (*Antheraea yamamai*, GenBank accession no. AB196795) (Fig. 3); and *Cyp9T1*, *Cyp9T2* (*Ips pini*, GenBank accession DQ676820), *Cyp9L1* (*A. gambiae* GenBank accession no. AF487781), and *Cyp9A13* (*Mamestra brassicae*, GenBank accession no. AY390260) (Fig. 4) are presented in four separate alignments according to differences in overall sequence relatedness between the four groups. That is, *Cyp9T1* is in a different family from the other genes in this study; *Cyp4BE1* is shorter than the other genes, but is similar to other known cytochromes P450; and *Cyp4G27* is longer than the other genes, but is otherwise very similar to other Cyp4G subfamily genes.

The characteristic Cyp4 family 13-amino acid motif EVDTFMFEGHDTT (Bradfield *et al.*, 1991; Werck-Reichhart & Feyereisen, 2000; Liu & Zhang, 2004) is present in all of the full-length deduced amino acid sequences of the Cyp4 genes (Figs 1–3) with two amino acid substitutions in Cyp4AY1 (residue 5, F to V; residue 8, E to A) (Fig. 1), one amino acid substitution each in Cyp4AY2 and Cyp4BH1 (residue 8, E to A in both cases) (Fig. 1), and two amino acid substitutions in Cyp4G27 (residue 1, E to Q; residue 5, F to I) (Fig. 3). In the latter case, the substitutions exactly

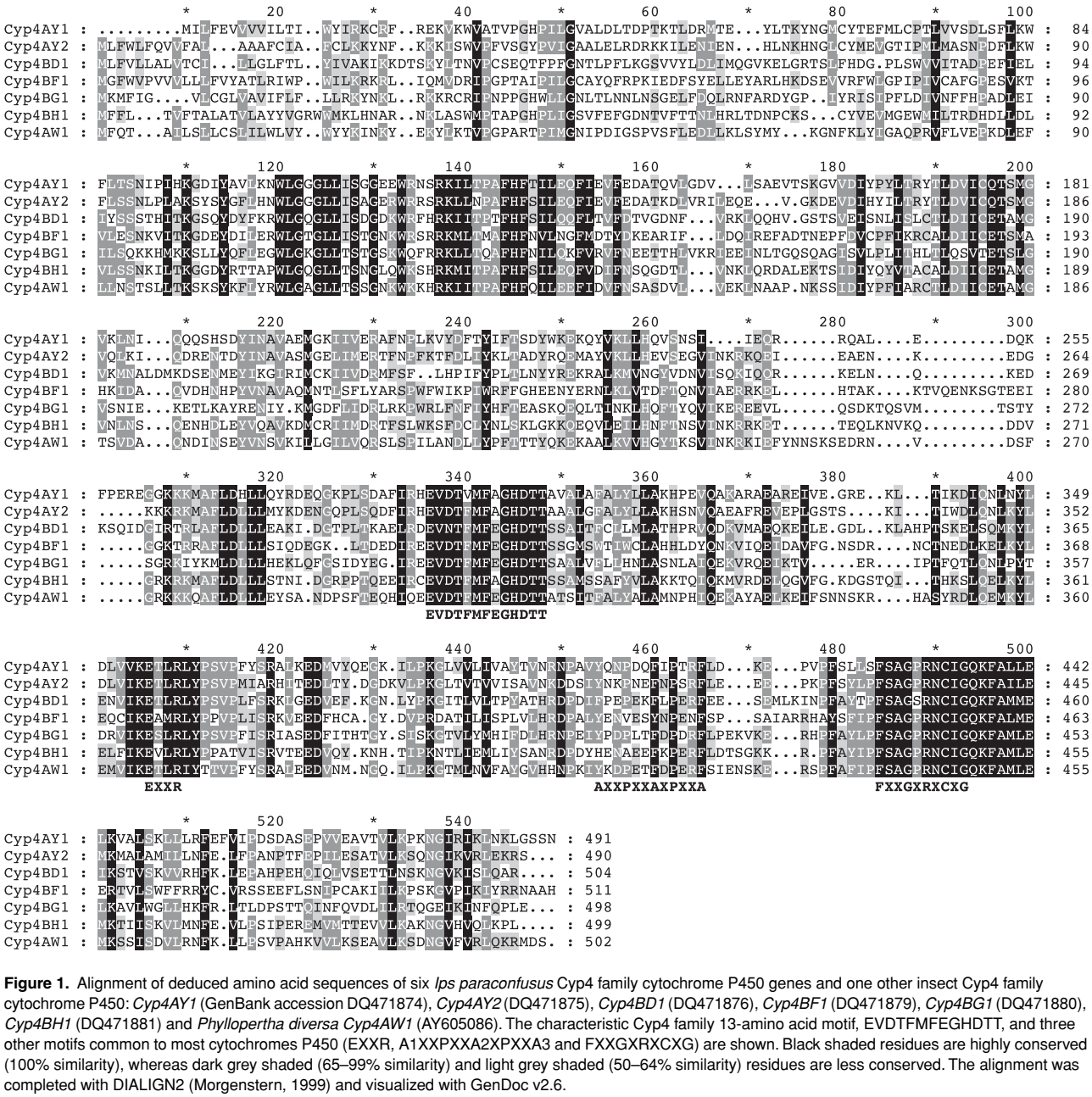

Figure 1. Alignment of deduced amino acid sequences of six *Ips paraconfusus* Cyp4 family cytochrome P450 genes and one other insect Cyp4 family cytochrome P450: *Cyp4AY1* (GenBank accession DQ471874), *Cyp4AY2* (DQ471875), *Cyp4BD1* (DQ471876), *Cyp4BF1* (DQ471879), *Cyp4BG1* (DQ471880), *Cyp4BH1* (DQ471881) and *Phyllopertha diversa Cyp4AW1* (AY605086). The characteristic Cyp4 family 13-amino acid motif, EVDTFMFEGHDTT, and three other motifs common to most cytochromes P450 (EXXR, A1XXPXXA2XPXXA3 and FXXGXRXCXG) are shown. Black shaded residues are highly conserved (100% similarity), whereas dark grey shaded (65–99% similarity) and light grey shaded (50–64% similarity) residues are less conserved. The alignment was completed with DIALIGN2 (Morgenstern, 1999) and visualized with GenDoc v2.6.

match substitutions reported in other known Cyp4 family genes of insects (Fig. 3). This motif is also present in the other four partial Cyp4 family cDNAs in this study (data not shown). It is not present, as expected in Cyp9 family genes, in Cyp9T1 (Fig. 4).

The highly conserved motif EXXR, thought to be involved in stabilization of the core of P450s (Werck-Reichhart & Feyereisen, 2000), is present in unmodified form in all of the full-length Cyp4 family deduced amino acid sequences (Figs 1–3). It is also present in all of the other partial Cyp4 cDNAs in this study (data not shown) and in the partial Cyp9 (Fig. 4).

The aromatic- and proline-containing 12 amino acid motif A1XXPXXA2XPXXA3, frequently found in P450s (Gotoh & Fujii-Kuriyama, 1989; Liu & Zhang, 2004), is present in all of the full-length Cyp4 family deduced amino acid sequences (Figs 1–3). It occurs in modified form in Cyp4BE1 (A1 to H, extra residue at position 9, Fig. 2), Cyp4BF1 (P4 to V, Fig. 1), Cyp4BH1 (A1 to H, P4 to A, Fig. 1), and Cyp4G27 (residues 1–5 modified, Fig. 3). It is also present in all of the other partial Cyp4 cDNAs in this study (data not shown) and in the partial Cyp9 (Fig. 4).

The highly characteristic 10 amino acid motif, FXXGXRX-CXG, containing the cysteine thought to be involved in

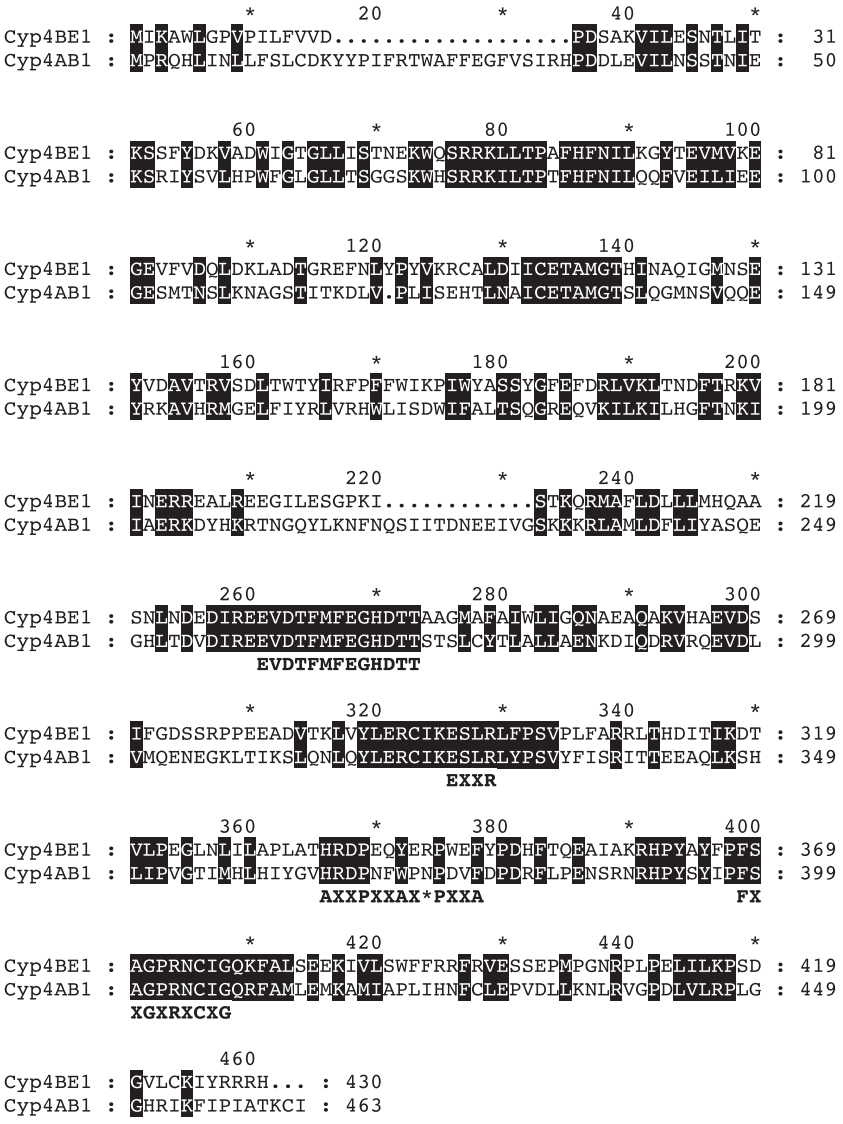

Figure 2. Alignment of deduced amino acid sequences of *Ips paraconfusus Cyp4BE1* (GenBank accession DQ471877) with that of *Solenopsis invicta Cyp4AB1* (AY345970). The characteristic Cyp4 family 13-amino acid motif, EVDTFMFEGHDTT, and three other motifs common to most cytochromes P450 (EXXR, A1XXPXXA2XPXXA3 and FXXGXRXCXG) are shown. The extra residue in A1XXPXXA2XPXXA3, present in both sequences, is denoted by an *. Black shaded residues are highly conserved (100% similarity). The alignment was completed with DIALIGN2 (Morgenstern, 1999) and visualized with GenDoc v2.6.

haem binding in P450s (Feyereisen, 1999, 2005; Werck-Reichhart & Feyereisen, 2000) is present in unmodified form in all of the full-length Cyp4 family deduced amino acid sequences (Figs 1–3). It is also present in all of the other partial Cyp4 cDNAs in this study (data not shown) and in the partial Cyp9 (Fig. 4).

Choice of housekeeping genes for gene expression profiling in Ips paraconfusus

Both of the conventional housekeeping gene candidates (*18S rRNA* and *β-actin* = *ACTB*) that we tested for gene expression profiling were present in all samples of treated and untreated male and female beetles (Supplementary material, Table 1). *18S rRNA* was found to show the least standard deviation across the groups and was therefore considered to be a more stably transcribed HKG than *ACTB*. In addition, target genes, if they are more stably transcribed than genes normally used as HKGs, can be used as alternative HKGs. We therefore also included the target genes in the validation process and found that *Cyp4G27* was even more stably transcribed than *18S rRNA* and was present in 71 of 72 samples (Supplementary material, Table 1), unlike *IparaCyp4A5*, which was stably expressed, but quite variably present across the samples (Supplementary material, Table 1).

Expression patterns of HMGR *and P450s in* Ips paraconfusus *following feeding in host tissue*

In most cases in which we recovered full-length cDNAs by RACE, the corresponding transcripts were detected by real-time PCR in almost all samples (> 94%) regardless of sex or time point (Supplementary material, Table 1). The only exception to this was *Cyp4BF1*, which was detected in 58.3% of male samples and 66.7% of female samples

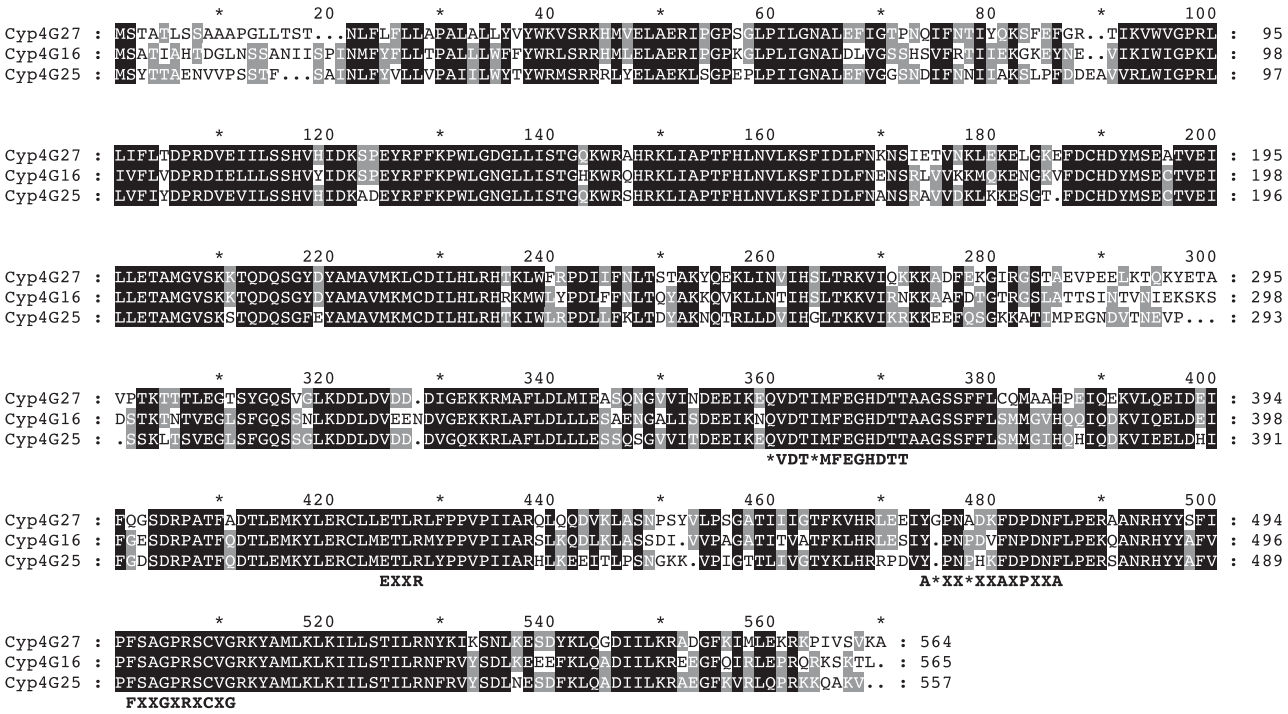

Figure 3. Alignment of deduced amino acid sequences of *Ips paraconfusus Cyp4G27* (GenBank accession DQ471883) and two other insect Cyp4G subfamily cytochromes P450: *Anopheles gambiae Cyp4G16* (AY062189) and *Antheraea yamamai Cyp4G25* (AB196795). The characteristic Cyp4 family 13-amino acid motif, EVDTFMFEGHDTT, containing two substitutions (*) that are perfectly conserved in all sequence, is shown. The motif A1XXPXXA2XPXXA3 also contains two substitutions (*) in *Cyp4G27*, but they are not conserved among the other sequences. Two other motifs common to most cytochromes P450 (EXXR and FXXGXRXCXG) are also shown. Black shaded residues are highly conserved (100% similarity), whereas grey shaded residues are less conserved (66–99% similarity). The alignment was completed with DIALIGN2 (Morgenstern, 1999) and visualized with GenDoc v2.6.

(59.2% of all samples). Each transcript was detected in a similar number of samples in both males and females, and every transcript was detected in at least 46.1% of samples (Supplementary material, Table 1).

Transcripts for the key mevalonate pathway enzyme HMG-CoA reductase (*HMGR*) accumulated in males, and not females, after feeding (Fig. 5). Transcript levels were approximately 109 times and 108 times higher at 8 h and 24 h following feeding, respectively, than in unfed males (0 h). The expression of *HMGR* in *I. paraconfusus* in response to hormone treatment and feeding has been characterized (Tillman *et al.*, 2004) and the general pattern observed in this experiment matches previously reported data (i.e. significant transcript accumulation in males after feeding). Thus, we felt confident that our treatment of the insects was similar to that in previously published reports and would be efficacious in inducing changes in transcript accumulation of other genes involved in early host colonization in *I. paraconfusus*.

Transcripts of two Cyp4 family cytochromes P450 accumulated significantly after feeding in males only (Fig. 6). *Cyp4AY1* transcripts increased 28.6 × and 39.6 × at 8 h and 24 h, respectively, whereas *Cyp4BD1* transcripts increased 5 × and 2.8 × at 8 h and 24 h, respectively, all compared with unfed individuals. Transcript levels of three cytochromes P450 declined significantly after feeding in males only. *Cyp4BE1* transcripts declined 11.7 × and 24.5 × at 8 h and 24 h, respectively, and *Cyp4BE2* transcripts declined 29.9 × at 24 h, compared with transcript levels in unfed individuals. *Cyp4BF1* transcripts declined 61.2 × at 8 h compared with transcript levels in unfed individuals. There were no instances in which there was differential transcript accumulation after feeding in females, but in which there was no change in males.

Transcripts of two Cyp4 family cytochromes P450 accumulated significantly after feeding in both males and females (Fig. 6). *Cyp4AY2* transcripts increased 2.5 × and 1.9 × at 8 h and 24 h, respectively, in males, and 4.5 × at 8 h in females, all compared with unfed individuals. *Cyp4BG1* transcripts increased 2.5 × and 6.3 × at 8 h in males and females, respectively, both compared with unfed individuals. Transcript levels of one cytochrome P450 declined significantly in both males and females following feeding (Fig. 6). *Cyp4BH1* transcripts declined 3.6 × at both 8 h and 24 h in males and 6.5 × in females at 24 h, all compared with unfed individuals. In one case, *IparaCyp4A5*, transcripts accumulated significantly in females (8.9 × and 2.1 × at 8 h and 24 h, respectively) and declined significantly in males (2.0 × and

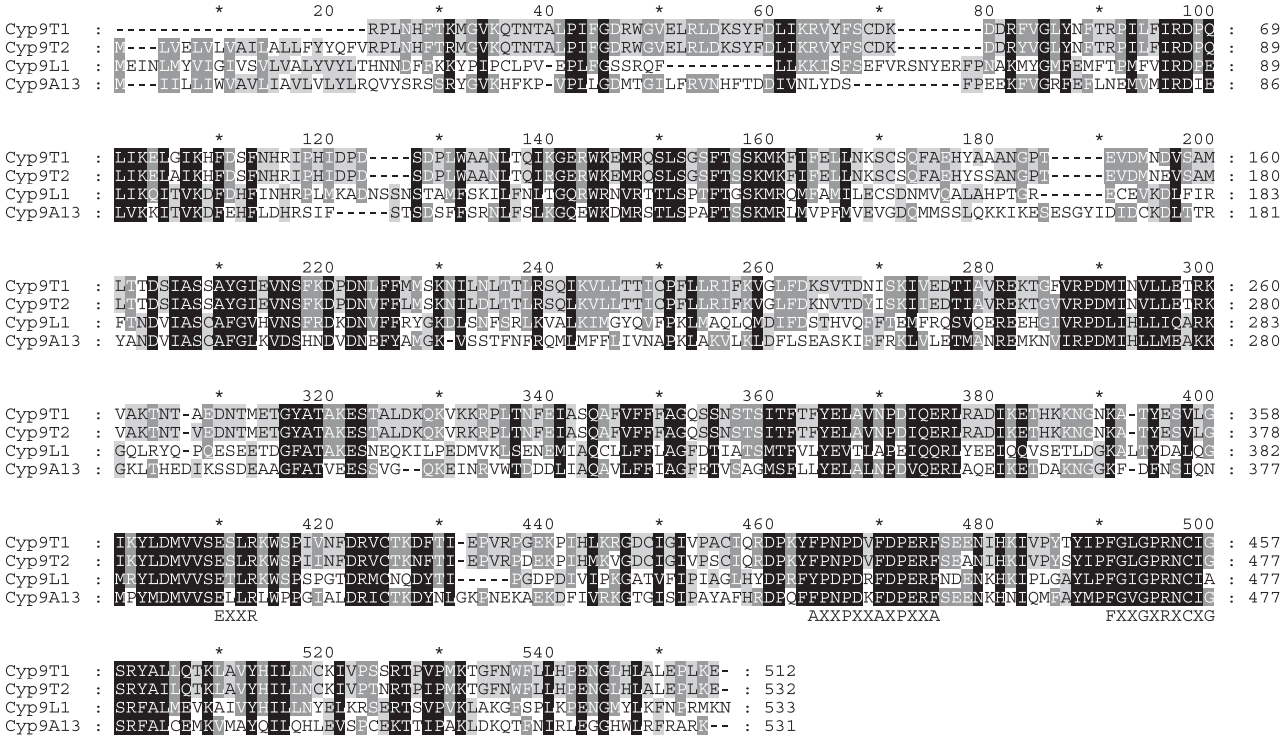

Figure 4. Alignment of deduced amino acid sequences of *Ips paraconfusus Cyp9T1* (GenBank accession DQ471884) with those of *Ips pini Cyp9T2* (DQ676820), *Anopheles gambiae Cyp9L1* (AF487781) and *Mamestra brassicae Cyp9A13* (AY390260). Three motifs common to most cytochromes P450 (EXXR, A1XXPXXA2XPXXA3 and FXXGXRXCXG) are shown. Black shaded residues are highly conserved (100% similarity), whereas dark grey shaded (75–99% similarity) and light grey shaded (50–74% similarity) residues are less conserved. A 22–25 amino acid portion of the Cyp9T1 N-terminal sequence, corresponding to the 5′-end of the unrecovered cDNA, is not included in this alignment. The alignment was completed with DIALIGN2 (Morgenstern, 1999) and visualized with GenDoc v2.6.

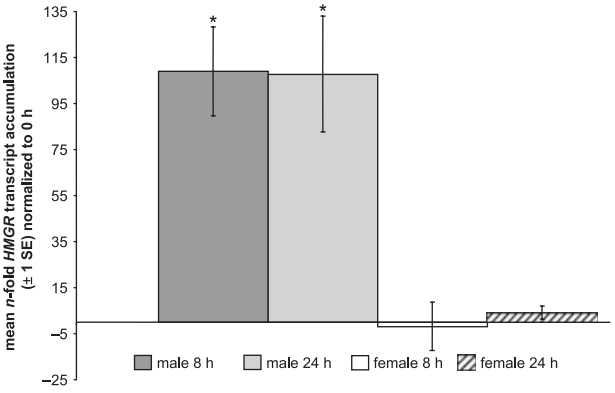

Figure 5. Mean *n*-fold accumulation (± 1 SE) of *HMG-CoA reductase* (*HMGR*) transcripts, over 0 h levels, in male and female *Ips paraconfusus* following feeding on host *Pinus ponderosa* phloem for 8 h and 24 h ($n = 12$). Males and females in the 0, 8 and 24 h feeding treatment groups were all the same age at the time of RNA extraction. Transcript levels were analysed with TaqMan® quantitative real-time PCR. Asterisks denote transcript accumulations that are significantly different than those observed at 0 h ($P < 0.05$, based on comparisons of 95% confidence intervals). Accumulation of this transcript in males following feeding on host tissue has been previously characterized (Tillman *et al.*, 2004) and the trend toward increased expression with feeding on the host validates the treatment method used in our experiments.

6.1 × at 8 h and 24 h, respectively), compared with unfed individuals (Fig. 6). For the remaining Cyp4 family P450s, feeding did not have a significant effect on transcript accumulation, as was also the case for *Cyp31B1*, the proposed nematode P450 (Fig. 6).

In the most dramatic example of transcript accumulation following feeding in this study, transcripts of *Cyp9T1* increased 85 030 × and 25 938 × at 8 h and 24 h, respectively, in males, and approximately 152 × in females at 24 h following exposure to host phloem, all compared with unfed controls (Fig. 7).

Discussion

Ips paraconfusus develops beneath the bark of the tops of large standing pine trees, the main stems of small standing trees, or in fallen trees or woody tree debris on the forest floor. Following maturation, new adults emerge through the bark and disperse by flight through the forest to search for and colonize new susceptible pines or recently fallen pine debris. After locating host material, adult *I. paraconfusus*, like many other species of bark beetles, quickly begin to feed on the phloem tissue, both for sustenance and in order to excavate a gallery system for reproduction. The males of

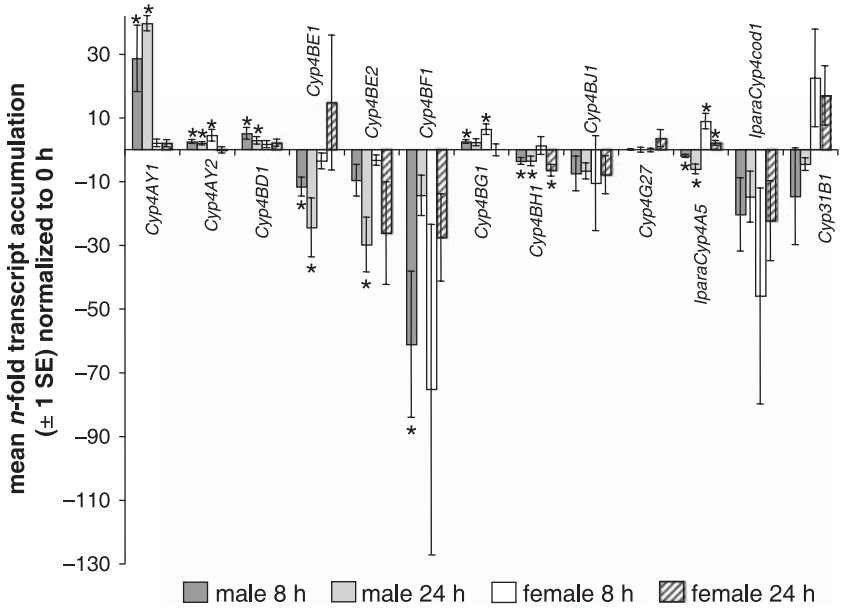

Figure 6. Mean *n*-fold accumulations (± 1 SE) of 12 Cyp4 family and one Cyp31 family cytochromes P450 transcripts, over 0 h levels, in male and female *Ips paraconfusus* following feeding on host *Pinus ponderosa* phloem for 8 h and 24 h ($n = 12$). Males and females in the 0, 8 and 24 h feeding treatment groups were all the same age at the time of RNA extraction. Transcript levels were analysed with TaqMan® quantitative real-time PCR. Asterisks denote transcript accumulations that are significantly different than those observed at 0 h ($P < 0.05$, based on comparisons of 95% confidence intervals).

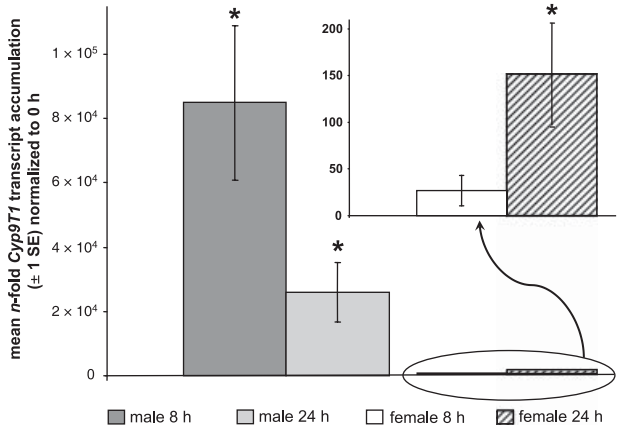

Figure 7. Mean *n*-fold accumulation (± 1 SE) of *Cyp9T1* transcripts, over 0 h levels, in male and female *Ips paraconfusus* following feeding on host *Pinus ponderosa* phloem for 8 h and 24 h ($n = 12$). Males and females in the 0, 8 and 24 h feeding treatment groups were all the same age at the time of RNA extraction. Transcript levels were analysed with TaqMan® quantitative real-time PCR. Asterisks denote transcript accumulations that are significantly different than those observed at 0 h ($P < 0.05$, based on comparisons of 95% confidence intervals). Accumulation of *Cyp9T1* transcripts in fed males was two to three orders of magnitude higher than that in females, so a smaller, inset graph is included to illustrate the significant response noted in females following feeding.

this species arrive at the host material first (the 'pioneering' sex) and excavate a small nuptial gallery under the bark. Feeding on phloem tissue during gallery excavation stimulates the production of pheromone components by the male (Tillman *et al.*, 2004) and up to three females are attracted to each male in the nuptial chamber (Furniss & Carolin, 1977). After mating with the male, the females excavate extensive linear galleries through the phloem, which is laden with terpenoids and other secondary metabolites (Seybold *et al.*, 2006). The females lay eggs along the sides of their galleries while the male guards the entrance to the gallery system. The feeding and excavating activities of the adults in standing trees, along with the pathogenic effects of fungi that they inoculate into the tree, often 'top-kill' what seem to be otherwise healthy trees.

Previous research with bark beetles (White *et al.*, 1979; Hunt & Smirle, 1988; Sandstrom *et al.*, 2006) has suggested that insect cytochrome P450 activity may be important in conversions of host monoterpenoids to pheromone components and other more readily excretable products. Our highly replicated and statistically analysed results show conclusively and directly that a short period of feeding in phloem by both males and females induces rapid and dramatic changes in gene expression in many members of the cytochrome P450 super-family (10 of the 13 bark beetle genes in this study), and that differential gene expression patterns occur, to some extent, between the sexes of *I. paraconfusus*. Sex-specific expression of cytochromes P450 has been reported in at least three other species of insects, *I. pini* (Sandstrom *et al.*, 2006); the German cockroach, *Blattella germanica* L. (Blattodea: Blattellidae) (Wen & Scott, 2001); and the fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Kasai & Tomita, 2003). The differential patterns of expression of cytochromes P450 in the sexes of *I. paraconfusus* at different points following contact with host phloem point to potentially different functions in the sexes during colonization of the host tree and subsequent reproductive activity.

The most dramatic change in transcript accumulation following feeding was observed in the case of *Cyp9T1*. The increase in males at both time points after feeding reflected

four and nearly five orders of magnitude in response. The mass of the principal pheromone component ipsenol produced by male *I. paraconfusus* in 1 week is a remarkable one-tenth of the mass of an individual insect (Seybold *et al*., 1995). Thus, pheromone biosynthesis may likely be accompanied by extreme changes in gene expression for the biosynthetic enzymes. Female *I. paraconfusus* also showed significant transcript accumulation for *Cyp9T1*, but this was apparent only at the later time period and was two orders of magnitude less than that observed in the males at either time point.

An interspecific homolog to *Cyp9T1* (*Ips pini Cyp9T2*) has been functionally characterized by Sandstrom *et al*. (2006). Our partial length *Cyp9T1* and the full length *Cyp9T2* have 94% identity (Table 1, Fig. 4); *Cyp9T2* expression is up-regulated 28 × in male *I. pini* midgut tissue following feeding for 32 h; and *Cyp9T2* expression is not up-regulated in female midgut tissue (Sandstrom *et al*., 2006). There was a > 500-fold difference between male and female basal levels of expression of the EST progenitor of *Cyp9T2* and the expressed protein for *Cyp9T2* catalysed the conversion of the monoterpene myrcene to ipsdienol (Sandstrom *et al*., 2006).

Our transcript accumulation pattern for *Cyp9T1* and the recent characterization of *Cyp9T2* from *I. pini* suggest that *Cyp9T1* is involved in male-specific aggregation pheromone production in *I. paraconfusus*. In *I. paraconfusus* one of the most likely final steps in the biosynthesis of ipsdienol is the oxidation of myrcene (Hendry *et al*., 1980) or, alternatively, oxidation of geranyl diphosphate (GDP). Myrcene, which males convert *in vivo* to ipsdienol (and eventually to ipsenol), is obtained in the insects' diet (Byers & Birgersson, 1990) and may be biosynthesized by the insect (Martin *et al*., 2003) from GDP (Gilg *et al*., 2005). At high headspace concentrations myrcene is toxic to *I. paraconfusus* (Byers *et al*., 1979), so both sexes would need to detoxify the compound from the host, but only males would need to remove it extremely quickly from their own bodies due to endogenous production, highlighting a possible requirement for substantial expression of the protein required to convert toxic myrcene to excretable ipsdienol (Vanderwel, 1994). In addition, males should be selected to produce copious amounts of aggregation pheromone very quickly upon feeding upon host phloem, as they need to both attract other conspecifics to help them to overcome the tree's defences and to attract females as mates.

Females are likely only concerned with detoxification of myrcene, rather than pheromone production. Because they spend some time mating with the male, after they arrive at the tree and before they begin to excavate egg galleries through the phloem, a protein required to detoxify myrcene by converting it to an excretable form would not need to be expressed in a substantial amount until several hours after entering the tree. In addition, because the females are required to clear myrcene from their diet, rather than biosynthesizing it in their own tissues where it could cause more damage, a lower, yet substantial, level of expression than that recorded in males should be expected. If this is the case, then, given the functional analysis of *Cyp9T2* by Sandstrom *et al*. (2006) and the differences in female expression of *Cyp9T1* and *Cyp9T2*, it seems reasonable to hypothesize that a sensitive analysis of the headspace within female galleries would reveal trace amounts of ipsdienol or other oxygenated products of myrcene in *I. paraconfusus*, but perhaps not in *I. pini*.

Two other cytochrome P450 genes in this study, *Cyp4AY1* and *Cyp4BD1*, exhibited transcript accumulation patterns that are consistent with being associated with pheromone biosynthesis in males. Both genes showed significant transcript accumulation in males, but no significant changes in females, at both time points after feeding. This pattern is indicative of male-specific physiological activity, such as pheromone production, or of juvenile hormone biosynthesis (Helvig *et al*., 2004), which is required for the induction of pheromone biosynthesis (Tillman *et al*., 1998, 2004).

As in the case of *Cyp9T1*, *Cyp4AY2* and *Cyp4BG1* showed significant transcript accumulation in both sexes following feeding. However, in neither case was the increase in transcripts, compared with 0 h levels, nearly as dramatic as that observed for *Cyp9T1*. The similar changes in transcript levels in both sexes, particularly for *Cyp4BG1*, which showed similar significant increases at 8 h, only, following feeding on host phloem, indicate that the protein products of these genes have similar functions in both sexes. Potential functions therefore include detoxification of host secondary metabolites (Feyereisen, 1999, 2005), biosynthesis of hormones (Tillman-Wall *et al*., 1992; Blomquist *et al*., 1994; Warren *et al*., 2002) used by both sexes in reproductive activities, or degradation of hormones (Sutherland *et al*., 1998) no longer needed by either sex. These gene products could also be involved in biosynthesis of another pheromone component, *cis*-verbenol, which is produced by both sexes, likely from the monoterpene α-pinene (Seybold *et al*., 2006). Transcript levels of *IparaCyp4A5* decreased significantly in males but increased significantly in females. Such a pattern may be indicative of a gene that is involved in female-specific JH or ecdysone production (Tillman-Wall *et al*., 1992; Blomquist *et al*., 1994; Warren *et al*., 2002) as the female prepares for reproductive activity as she enters the host tree. Another possibility is that this gene is involved in detoxification of specific secondary metabolites that the female may encounter in the phloem resin during egg gallery extension.

One gene, *Cyp4G27*, was constitutively expressed in both sexes both before and after feeding on host phloem and was detected in 71 of the 72 total samples (Supplementary material, Table 1). The protein product of this gene may be involved in detoxification of constitutive host secondary

metabolites, as it would likely be present in the beetle at all times during and just following dispersal and colonization. Such a gene may also be involved in some aspect of basic metabolism required for flight muscle degradation, which begins rapidly in both sexes upon initial contact by the beetle with the tree (Borden & Slater, 1969; Bhakthan *et al.*, 1970). The stable expression of *Cyp4G27* under our experimental conditions allowed us to use it as an HKG in our study. This gene or its homologues may be valuable for accurate assessment of cytochrome P450 expression in *I. paraconfusus* and other bark beetles instead of the less stably expressed *ACTB*, which has been used in real-time PCR studies with *I. pini* fed on host tissue (Keeling *et al.*, 2004; Sandstrom *et al.*, 2006), or other commonly used HKGs, such as *18S rRNA*.

The levels of induced expression of genes related to pheromone biosynthesis in pine bark beetles have generally ranged from two- to 30-fold (Tittiger *et al.*, 1999, 2003; Keeling *et al.*, 2004, 2006; Tillman *et al.*, 2004; Sandstrom *et al.*, 2006). In some instances, relative expression patterns have been modulated similarly in both the pheromone- and nonpheromone producing sex. However, the pheromone-producing sex likely has higher basal levels of activity or expression than the opposite sex (Martin *et al.*, 2003; Tillman *et al.*, 2004; Keeling *et al.*, 2004, 2006). The relative changes in expression of *Cyp9T1* by male *I. paraconfusus* in this study far exceed by several orders of magnitude all previously reported levels for pheromone biosynthetic genes. For example, feeding-induced expression of *Cyp9T2* in male *I. pini* midgut tissue (2 × at 8 h, 28 × at 32 h) (Sandstrom *et al.*, 2006) and *Cyp9T1* in male *I. paraconfusus* whole body extracts (85 000 × at 8 h, 26 000 × at 24 h) (this study) were extremely different. Also, our measurements of > 100-fold feeding-induced expression of *HMGR* in male *I. paraconfusus* exceed previous reports [approximately sevenfold for male *I. paraconfusus* (Tillman *et al.*, 2004) and approx. 15-fold for male *I. pini* (Keeling *et al.*, 2004)]. We attribute these differences in part to improvements in the quantitative analysis of gene expression, which include improvements in the extraction of RNA, the use of more stable HKGs, and the use of real-time PCR. Other reasons for differences in expression level may involve inherent differences in the regulation of pheromone biosynthesis in *I. paraconfusus* and *I. pini* (Tillman *et al.*, 2004); the timing of the feeding treatments; or the use of whole body or thoracic tissue (Tillman *et al.*, 2004 and this study) vs. midgut tissue (Keeling *et al.*, 2004; Sandstrom *et al.*, 2006) as the source for the RNA. The choice and stability of the HKG in the analysis is a factor that merits specific consideration. In our analysis the signal detected from *ACTB* in male *I. paraconfusus* fluctuated by 4 and 2 threshold cycle (C_T) values at the 8 and 24 h measurements when compared with the 0 h measurement. Thus, assuming similar behaviour in male *I. pini*, the use of *ACTB* (a relatively unstable HKG) may have led to underestimated increases in the expression of *Cyp9T2* (Sandstrom *et al.*, 2006) and *HMGR* (Keeling *et al.*, 2004) in previous real-time PCR-based feeding studies. Furthermore, TaqMan PCR (vs. SYBR Green-based PCR) provides greater analytical sensitivity, which may have allowed the detection of increased expression of *Cyp9T1* in female *I. paraconfusus*. Thus, the real-time TaqMan PCR technique described herein has led to sensitive measurements of gene expression with relatively controlled levels of variability among replicated samples. Instances of higher variability for the expression of some P450s are likely explained by smaller sample sizes afforded when signal was absent from a subset of the 12 original replicates (Fig. 6, Supplementary Table 1).

Of the 13 cytochrome P450 genes described in this study, 10 showed differential transcript accumulation following feeding in males alone (five genes) or in both males and females. Cytochrome P450 genes therefore seem to play a major role in the physiological and behavioural changes in *I. paraconfusus* during host colonization. Differential transcription or transcript stability of this subset of cytochrome P450 genes was particularly pronounced in males, both in terms of the number of genes that showed a response and in terms of the magnitude of the response in several cases, most notably *Cyp9T1*. Further gene discovery, expression analyses, and functional characterization of cytochromes P450 in *I. paraconfusus* and other bark beetles will provide new information about the ecology of these significant insects and has the potential to allow the development of new pest management tools based upon the inhibition of specific gene products.

Experimental procedures

Insects and host material

Immature *I. paraconfusus* were collected in October 2003 and June and July 2004 in infested ponderosa pine, *Pinus ponderosa*, at the University of California Blodgett Forest Research Station (El Dorado Co., California, ≈ 1200 m elevation; 38°53′11′N 120°38′48′W). Infested material was placed in wooden emergence cages in the lab (Browne, 1972) and adults that emerged were collected daily. Fresh *P. ponderosa* was collected at the same times at the same location. Small holes, slightly larger than the diameter of a beetle body, were drilled through the outer bark and just into the phloem of fresh pine logs. Male and female beetles were separated from each other by the sound-producing organ, the *pars stridens*, present only in males (Lanier & Cameron, 1969). The sexes were separately inserted into the holes, one beetle per hole. The insects were retained there with screening and allowed to feed for ≈ 20 h before they were removed. Upon removal, the insects were flash frozen in liquid nitrogen and were stored at −80 °C for later RNA isolation.

RNA isolation and cDNA synthesis

About 23 beetles were used in each extraction and males were extracted separately from females. Frozen beetles were ground in liquid nitrogen with a mortar and pestle and were further homogenized

with a Duall grinder (Kimble/Kontes, Vineland, NJ, USA). Isolation of total RNA was conducted following the protocol provided in the RiboPure® kit from Ambion, Inc. (Austin, TX, USA). cDNA was synthesized from total RNA following the protocol provided with the RETROScript® kit (Ambion, Inc.) and using the random decamers provided with the kit.

Degenerate polymerase chain reaction, cloning and sequence analyses

Synthesized cDNA was used as the template and Taq DNA polymerase (New England Biolabs Inc., Beverly, MA, USA), Platinum® Taq DNA Polymerase High Fidelity (Invitrogen Corp., Carlsbad, CA, USA), or SuperTaq™ DNA polymerase (Ambion, Inc.) were used in all degenerate PCR reactions. All primers were obtained from Operon Biotechnologies, Inc. (Huntsville, AL, USA). Thermocyclers used for these and all other PCR reactions in this study (other than real-time PCR) were an Eppendorf Mastercycler gradient and a Perkin Elmer GeneAmp 2400 (Perkin Elmer, Norwalk, CT, USA).

To obtain a fragment of an *I. paraconfusus* β-actin gene for use as a potential housekeeping gene (HKG) in subsequent real-time PCR analyses, degenerate primers Act-1 and Act-3R (0.125 μM each) (Supplementary material, Table 2) (Voigt & Wöstemeyer, 2000) were used with fed female *I. paraconfusus* cDNA template with the following temperature programme: 5 min at 95 °C; 30 cycles of 1 min at 94 °C, 25 s at 58 °C, 1 min at 72 °C; 5 min at 72 °C. The ≈ 500 bp product was cloned into pCR®2.1-TOPO® with a TOPO TA Cloning® kit (Invitrogen) and the cloning reaction was transformed into *Escherichia coli* chemically competent TOP10 cells (Invitrogen). Transformants were blue-white selected on kanamycin/LB/X-gal plates, a single colony was grown overnight in kanamycin/LB broth and plasmid DNA was extracted with a QIAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA, USA). The inserts were sequenced at the University of California, Davis Division of Biological Sciences Automated DNA Sequencing Facility and were compared with known sequences in GenBank by BLASTp (Altschul *et al.*, 1990). Sequences of inserts from four clones were compared, and all were identical.

Three pairs of degenerate primers were used to screen cDNA derived from fed male and female *I. paraconfusus* for candidate P450s. Primer set Cyp4Af/Cyp4Ar and primer set Cyp4Bf/Cyp4Br (Supplementary material, Table 2), were designed and used in PCR following the method of Snyder *et al.* (1996). The third set of primers, Cyp4codF/Cyp4codR (Supplementary material, Table 2), was designed with CODEHOP (Rose *et al.*, 1998) from the following insect P450 protein sequences obtained from GenBank [accession numbers (species)]: AAP94193 (*Tribolium castaneum*), AAP94192 (*T. castaneum*), AAF67724 (*Diabrotica virgifera virgifera*), XP_311065 (*Anopheles gambiae*), P29981 (*Blaberus discoidalis*), AAC03111 (*Coptotermes acinaciformis*), and AAP97090 (*Solenopsis invicta*)]. As with the other two primer sets, Cyp4codF/Cyp4codR (1 μM each) were used in degenerate PCR with cDNA derived from fed male and female *I. paraconfusus,* and the following temperature programme was utilized: 2 min at 95 °C; six cycles of 30 s at 95 °C, 30 s at 65 °C –60 °C (–1 °C per cycle), 1 min at 72 °C; 35 cycles of 30 s at 95 °C, 30 s at 57 °C, 1 min at 72 °C; 10 min at 72 °C.

Products of all degenerate PCR reactions were visualized on agarose gels. Each reaction yielded a discrete band of ≈ 450 bp. The product of each reaction was cloned as previously described for β-actin.

The inserts of 41 clones derived from primer set Cyp4Af/Cyp4Ar, nine clones derived from primer set Cyp4Bf/Cyp4Br, and 37 clones derived from primer set Cyp4codF/Cyp4codR were sequenced. Insert-containing plasmids were sampled and sequenced until ≈ 9 consecutive samples did not yield novel P450-related sequence data (87 inserts total). Sequences were translated and deduced amino acid sequences from continuous ORFs were compared with known sequences in GenBank by BLASTp (Altschul *et al.*, 1990) to ensure that they encoded P450-like proteins.

All deduced amino acid sequences were aligned with ClustalW (Chenna *et al.*, 2003) and results were visualized with TreeView (Page, 1996). ClustalW and TreeView analyses revealed that a total of 13 novel P450s (12 Cyp4 family genes and one Cyp31 family gene) were represented by the 87 analysed inserts. Expression patterns of these 13 genes plus one Cyp9 family gene (see below) were explored by real-time PCR expression analyses. Deduced amino acid sequences were submitted to the P450 nomenclature committee and were given names based upon their criteria for classification of P450 genes (D. Nelson, Department of Molecular Sciences, University of Tennessee, personal communication). Names that appear here follow the committee's recommendations.

Cloning of a Cyp9 cDNA

Primer set Cyp9F2/Cyp9R1 (Supplementary material, Table 2) was designed directly from a putative Cyp9 P450 sequence (IPG-Contig-0128–1) from the closely related *I. pini*, which was available on a public EST database (http://bioinformatics.unr.edu/beetle/fasta/Ipini.txt). A standard PCR reaction using male *I. paraconfusus*-derived cDNA, 0.2 μM of each primer, and Platinum® Taq DNA Polymerase High Fidelity, was conducted with the following temperature programme: 3 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 42 °C, 2 min at 72 °C; 10 min at 72 °C. The product of the reaction, a discrete band of ≈ 1550 bp, was visualized on an agarose gel and was cloned and sequenced as previously described for β-actin cDNA. Four colonies were chosen, and the inserts were found to be identical to each other and very similar, by BLASTp analysis (Altschul *et al.*, 1990), to a number of known Cyp9 genes but different from the *I. pini* putative Cyp9 from which the primers were designed.

End sequence determination and cloning of full-length cDNAs

End sequence data were successfully obtained by 5′- and 3′-Rapid Amplification of cDNA Ends (RACE) for eight of the 13 products of the degenerate PCR reactions. Either or both 5′- and/or 3′-end sequence data for the other five Cyp4 cDNAs were not obtained following extensive efforts, nor was the 5′-end of the Cyp9. The BD SMART™ RACE cDNA Amplification Kit (BD Biosciences, Mountain View, CA, USA) was used, and the manufacturer's protocol and primer design recommendations were followed. Total RNA, obtained either as outlined above or isolated for work in real-time PCR analyses from both fed and unfed males and females (see below), was used in construction of 3′- and 5′-RACE templates. Products of RACE PCR were cloned as previously described for β-actin. Inserts were sequenced as described and were compared, with GeneStudio (GeneStudio, Inc., Suwanee, GA, USA), to the known sequence data for each gene to ensure contiguous sequence data. End sequence data derived from successful RACE allowed the development of primers (Supplementary material, Table 2) for the amplification of full-length coding regions of eight of the Cyp4 cDNAs.

Amplifications were carried out with 5′-RACE cDNA as the template source, Platinum® Taq DNA Polymerase High Fidelity,

and 0.2 μM of each primer. The temperature used in the annealing stage of the PCR reaction varied with the primer pair and the cycling parameters were as follows: 3 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at annealing temperature, 2 min at 72 °C; 10 min at 72 °C. Following each reaction, a single, discrete band, ≈ 1500 bp, was visualized on an agarose gel. The PCR products were cloned into pCR2.1TOPO and transformed into *E. coli* TOP10 cells. Plasmids were harvested with the QIAprep Spin Miniprep kit and inserts were sequenced on both strands. Sequence data for all 14 P450s and β-actin were deposited in GenBank (accession numbers DQ471873–DQ471887).

Analyses of cDNA sequence data

Deduced amino acid sequences were obtained with use of the ExPASy Translate Tool (http://www.expasy.org/tools/dna.html). Deduced amino acid sequences were compared with arthropod databases with BLASTp (Altschul *et al*., 1990) on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). The presence or absence of N-terminal targeting peptides and likely target locations if present were assessed with TargetP 1.01 (Emanuelsson *et al*., 2000) with specificity set at > 0.95. Deduced amino acid sequences were aligned with each other and with arthropod P450 sequences from databases with DIALIGN2 (Morgenstern, 1999) and were visualized with GenDoc (Nicholas *et al*., 1997).

Experimental treatments for expression analyses

Ips paraconfusus were collected and reared as described above (2004 collection). Immediately upon emergence, 60 males and 60 females were divided into three groups of 20 males and three groups of 20 females. One group of each sex was placed on new glass wool in glass Petri dishes and kept in a drawer for 24 h. The second group of each sex was placed immediately and individually into holes drilled in small freshly cut *P. ponderosa* logs, as described above. The third group of each sex was placed on glass wool in Petri dishes and kept in the drawer for 16 h, after which they were also placed individually into the same logs and allowed to feed for 8 h. This resulted in two sets of phloem-fed beetles for each sex: 24 h of feeding starting at 16.00 h on 20 September 2004 and 8 h of feeding starting at 08.00 h on 21 September 2004. All 80 beetles were removed from the logs at 16.00 h on 21 September 2004. Control insects, 0 h of feeding, were the 20 individuals of each sex that remained on the glass wool for the duration of the feeding treatments. Thus, beetles in the 0, 8 and 24 h feeding treatment groups were all the same age when the experiment was terminated. Following feeding and excision from the host or time on glass wool, all insects were checked for viability under a dissecting microscope and 12 were chosen from each sex and time point for use in real-time PCR analyses. Viable insects were cut sagittally with a fresh razor blade on fresh weighing paper and each insect was immediately transferred to an individual well in a 96-well plate containing 500 μl of stabilization solution (nucleic acid purification lysis buffer; Applied Biosystems, Foster City, CA, USA) and were stored at –20 °C until RNA extraction.

RNA extraction and cDNA synthesis for expression analyses

Proteinase K and two grinding beads (4 mm diameter, stainless steel beads, SpexCertiprep, Metuchen, NJ, USA) were added into the beetle lysate and then homogenized in a GenoGrinder 2000 (SpexCertiprep) for 2 min at 1000 strokes per min. Protein was digested at 56 °C for 30 min followed by a 30-min period at –20 °C to reduce foam. Total RNA was extracted from the tissue lysates using a 6700 automated nucleic acid workstation (Applied Biosystems) according to the manufacturer's instructions.

Transcript quantification

For each target gene, two primers and an internal, fluorescently labelled TaqMan probe [5′ end, reporter dye FAM (6-carboxyfluorescein), 3′ end, quencher dye TAMRA (6-carboxytetramethylrhodamine)] was designed using default parameters of Primer Express software 2.0 (Applied Biosystems). TaqMan PCR systems (Supplementary material, Table 3) were validated using defined protocols (Leutenegger *et al*., 1999).

cDNA was synthesized from 20 μl DNase (RNase-free DNase I, Invitrogen) digested total RNA using 100 units of SuperScript III, 600 ng random hexadeoxyribonucleotide (pd(N)6) primers (random hexamer primer) 10 U RNaseOut (RNase inhibitor), and 1 mM dNTPs (all Invitrogen) in a final volume of 40 μl. The reverse transcription reaction proceeded for 120 min at 50 °C. After addition of 60 μl of water, the reaction was terminated by heating for 5 min at 95 °C and cooling on ice.

Each PCR reaction contained 20 × primer and probes (Supplementary material, Table 2) for the respective TaqMan system with a final concentration of 400 nM for each primer and 80 nM for the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 μl of the diluted cDNA sample in a final volume of 12 μl. The samples were placed in 384-well plates and amplified in an automated fluorometer (ABI PRISM 7900 HTA FAST, Applied Biosystems). The manufacturer's standard amplification conditions were used: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Fluorescent signals were collected during the annealing period and CT values extracted with a threshold of 0.04 and baseline values of 3–15. For stronger signals, the baseline was adjusted manually to 3–10. *Ips paraconfusus* HMG-CoA reductase (*HMGR*, GenBank accession number AF071750) was used as a positive control because its expression pattern in this insect, following feeding on host phloem, is well established (Tillman *et al*., 2004).

Housekeeping gene validation experiment

In order to determine the most stably transcribed housekeeping gene, a housekeeping gene (*HKG*) validation experiment was run on all samples using a universal insect 18S rRNA (ssr RNA) TaqMan system, a β-actin (ACTB) TaqMan PCR system, and all of the *I. paraconfusus* Cyp4 and Cyp9 TaqMan PCR systems. *Cyp4G27* was the most stably transcribed gene under our conditions, so we used it as a HKG to normalize the raw data, except when analysing transcript levels of *Cyp4G27*, in which case we used 18S rRNA (the next most stably transcribed gene) as the HKG.

Relative quantification of gene transcript levels

Final quantification was performed using the comparative C_T method ($\Delta\Delta C_T$, User Bulletin no. 2, Applied Biosystems) and is reported as relative transcription or the *n*-fold difference relative to a calibrator cDNA (i.e. transcript levels at 0 h feeding for each sex). The relative

linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta CT}$. Therefore, all gene transcription is expressed as an *n*-fold difference relative to the calibrator. Transcript accumulation was considered to be significantly different from the calibrator (0 h level for any specific target gene) if the 95% confidence intervals of the mean did not overlap the mean level of the calibrator.

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Supplementary material

The following supplementary material is available for this article online:

Supplementary Table 1. *Detection of P450 and HMG-CoA reductase transcripts in quantitative PCR experiments by TaqMan® primer and probe sets designed for each cDNA*

Supplementary Table 2. *Primers used to obtain partial and full-length cytochrome P450 cDNAs and other cDNAs from Ips paraconfusus.*

Supplementary Table 3. *Primers and TaqMan® probes used in real-time PCR expression analyses.*

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