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Elucidation of the sex-pheromone biosynthesis producing 5,7-dodecadienes in *Dendrolimus punctatus* (Lepidoptera: Lasiocampidae) reveals Δ11- and Δ9-desaturases with unusual catalytic properties

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ABSTRACT

Sex pheromones produced by female moths of the Lasiocampidae family include conjugated 5,7-dodecadiene components with various oxygenated terminal groups. Here we describe the molecular cloning, heterologous expression and functional characterization of desaturases associated with the biosynthesis of these unusual chemicals. By homology-based PCR screening we characterized five cDNAs from the female moth pheromone gland that were related to other moth desaturases, and investigated their role in the production of the (*Z*)-5-dodecenol and (*Z*5,*E*7)-dodecadienol, major pheromone constituents of the pine caterpillar moth, *Dendrolimus punctatus*. Functional expression of two desaturase cDNAs belonging to the Δ11-subfamily, *Dpu*-Δ11$_1$-APSQ and *Dpu*-Δ11$_2$-LPAE, showed that they catalysed the formation of unsaturated fatty acyls (UFAs) that can be chain-shortened by β-oxidation and subsequently reduced to the alcohol components. A first (*Z*)-11-desaturation step is performed by *Dpu*-Δ11$_2$-LPAE on stearic acid that leads to (*Z*)-11-octadecenoic acyl, which is subsequently chain shortened to the (*Z*)-5-dodecenoic acyl precursor. The *Dpu*-Δ11$_1$-APSQ desaturase had the unusual property of producing Δ8 mono-UFA of various chain lengths, but not when transformed yeast were grown in presence of (*Z*)-9-hexadecenoic acyl, in which case the biosynthetic intermediate (*Z*9,*E*11)-hexadecadienoic UFA was produced. In addition to a typical *Z*9 activity, a third transcript, *Dpu*-Δ9-KPSE produced *E*9 mono-UFAs of various chain lengths. When provided with the (*Z*)-7-tetradecenoic acyl, it formed the (*Z*7,*E*9)-tetradecadienoic UFA, another biosynthetic intermediate that can be chain-shortened to (*Z*5,*E*7)-dodecadienoic acyl. Both *Dpu*-Δ11$_1$-APSQ and *Dpu*-Δ9-KPSE thus exhibited desaturase activities consistent with the biosynthesis of the dienoic precursor. The combined action of three desaturases in generating a dienoic sex-pheromone component emphasizes the diversity and complexity of chemical reactions that can be catalysed by pheromone biosynthetic fatty-acyl-CoA desaturases in moths.

1. Introduction

Mate finding in moths is typically mediated by the long-range attraction of males towards multi-component female sex-pheromones (Wyatt, 2003). Presumably, this fine-tuned communication system has evolved through the diversification of female emitted compounds and subsequent coordinated changes in the male olfactory system (Phelan, 1997; Cardé and Haynes, 2004). Pheromone biosynthesis is accounted for by a few key enzymes including β-oxidases and fatty-acyl desaturases prior to subsequent modifications of the carboxyl end by reductases, acetyl transferases and/or oxidases (Tamaki, 1985; Bjostad et al., 1987; Blomquist et al., 2005). Desaturases catalyse the insertion of double bonds into fatty-acyl chains, at a position determined by the enzyme specificity, and account for most of the structural variation found among pheromone precursors (Bjostad and Roelofs, 1983; Roelofs and Bjostad, 1984; Percy-Cunningham and MacDonald, 1987; Jurenka and Roelofs, 1993; reviewed in Tillman et al., 1999).

Desaturases that serve a role in moth reproduction constitute a dynamically evolving gene family whose extant members likely

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originated from an ancestral desaturase prior to the divergence of the dipteran and lepidopteran lineages (Knipple et al., 2002). Extant moth Δ9-desaturases can act either as metabolic desaturases (Liu et al., 1999) or be involved in the production of Δ9 unsaturated fatty acids (UFAs) used for pheromone production (*e.g.*, Hao et al., 2001, 2002; Rosenfield et al., 2001). Δ11-Desaturases evolved following the divergence of flies and moths (Liénard et al., 2008) and serve exclusively in pheromone production (*e.g.*, Knipple et al., 1998, 2002; Liu et al., 1999, 2004; Rosenfield et al., 2001; Jeong et al., 2003; Moto et al., 2004). The use of heterologous expression systems has proven to be a remarkable tool to study the desaturase functionalities and has demonstrated that in contrast to Δ9-desaturases, members of the Δ11-desaturase subfamily display a broad substrate-, stereo- and regio-specificity including *Z*10, *Z*11, *Z*/*E*11, *E*11, Δ11/10,12 and Δ11/Δ11,13 desaturase activities (*e.g.*, Roelofs and Rooney, 2003; Moto et al., 2004; Serra et al., 2007). Considerable innovations in the moth pheromonal system are thus likely accounted for by the evolution of a limited number of desaturase genes (Knipple et al., 1998; Liu et al., 2002a,b, 2004; Hao et al., 2001, 2002; Rosenfield et al., 2001; Roelofs et al., 2002; Moto et al., 2004; Rodríguez et al., 2004; Serra et al., 2006, 2007; Matoušková et al., 2007; Park et al., 2008; Liénard et al., 2008).

Conjugated dienes are typically formed by the immediate action of a bifunctional desaturase with isomerization around the first double bond or by two stepwise desaturation steps (Blomquist et al., 2005). Desaturases of the first type have been described in *Bombyx mori* (Bjostad and Roelofs, 1984; Yamaoka et al., 1984; Ando et al., 1988; Moto et al., 2004), *Cydia pomonella* (Löfstedt and Bengtsson, 1988), *Manduca sexta* (Fang et al., 1995; Matoušková et al., 2007) and *Spodoptera littoralis* (Martinez et al., 1990; Navarro et al., 1997; Rodríguez et al., 2002; Serra et al., 2006). In contrast, pheromone components found in *Thysanoplusia intermixta* (Ono et al., 2002), *Epiphyas postvittana* (Foster and Roelofs, 1990; Liu et al., 2002a) and *Lampronia capitella* (Liénard et al., 2008) are formed after two sequential desaturation steps. Biochemical studies supported that dienyl compounds found among the Lasiocampidae family are also likely to be synthesized by sequential desaturation (Zhao et al., 2004). Most Lasiocampidae species use sex pheromones with conjugated dodecadiene derivatives with double bond positions fixed at the 5th and 7th carbons, *i.e.*, a Δ5,Δ7-12:X chemical theme (*e.g.*, Underhill et al., 1980; Ando et al., 1982; Zhao et al., 1993; Klun et al., 2000; Rotundo et al., 2004; Haynes et al., 2007; Kong et al., 2007 and references therein). The sex-pheromone components of *Dendrolimus punctatus* consist of the (*Z*5,*E*7)-dodecadienol and the mono-unsaturated (*Z*)-5-dodecenol (Zhao et al., 1993). *In vivo* labelling experiments demonstrated that a first Δ11-desaturation takes place on C_{18} to form the *Z*11-18:acyl (Zhao et al., 2004). Labelled *Z*11-18:acyl was incorporated into *Z*5- and *Z*5,*E*7-12:acyl and the corresponding alcohols, indicating that the *Z*5-12:OH is likely formed by Δ11-desaturation followed by three cycles of β-oxidation and subsequent reduction (Zhao et al., 2004). Which of the three potential mono-unsaturated substrates, *i.e.*, *Z*9-16:acyl, *Z*7-14:acyl or *Z*5-12:acyl, is actually the substrate for the second desaturation producing the *E* double bond could not be conclusively evidenced *in vivo*. Therefore, it remained unknown whether one or several desaturases with different specificity with respect to chain length of the substrate and geometry of the products are actually implicated in pheromone production in this species.

In the present study, we further investigate the pheromone biosynthesis in *D. punctatus* and demonstrate that the production of *Z*5,*E*7-12:acyl may occur from two alternative biosynthetic routes involving either *E*11-desaturation of the *Z*9-16:acyl or *E*9-desaturation of the *Z*7-14:acyl, respectively, to produce the *Z*9, *E*11-16:acyl or *Z*7,*E*9-14:acyl, which can be subsequently chain-shortened and reduced to the *Z*5,*E*7-12:OH.

2. Materials and methods

2.1. Insect collection

Last larval instars and newly formed cocoons of *D. punctatus* were collected from pine trees in the vicinity of Yingshan (Hubei, China). Pupae were gently removed from their cocoons and sexed. Females were kept in a climate chamber under a reversed 17:7 light:dark cycle at 25 ± 1 °C until emergence.

2.2. Total RNA extraction and cDNA synthesis

The intersegmental PG membrane located between the 8th and the 9th abdominal segments was carefully dissected with ethanol-rinsed micro-scissors and separated from its internal fat bodies (FB). PG or FB tissues were immersed in RNA*later* reagent (Qiagen) and stored at −20 °C. Total RNA was isolated using the RNeasy Isolation kit (Qiagen AB, Solna, Sweden) including a cleaning step with DNAse in order to remove any genomic DNA. First-strand cDNA was synthesized by using 1 μg of total RNA and a reverse transcriptase (Stratascript) (Stratagene, AH Diagnostics, Skärholmen, Sweden).

2.3. Characterization of desaturase-encoding cDNAs

PG cDNA from 10 newly emerged females (day 0) was used as template in PCR amplifications using two different pairs of degenerated primers designed based on the conserved TAGAHR and GEGFH histidine-rich motifs of desaturases (Rosenfield et al., 2001; Roelofs et al., 2002). PCR reactions were performed in a PCR GeneAmp 9700 Thermo Cycler (Applied Biosystems, Stockholm, Sweden) using the AmpliTaq Gold chemistry (Applied Biosystems) and the following cycling conditions: 95 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min 30 s followed by a final extension step at 72 °C for 40 min. Specific PCR amplification products (~560 bp) were ligated into the pGEM®-T Easy vector system (Promega Biotech AB, Nacka, Sweden) and transformed into *Escherichia coli* DH5α cells (Invitrogen AB, Lidingö, Sweden). Plasmid DNAs were purified and positive clones were subjected to sequencing using universal M13 primers and the Big Dye Terminator cycle sequencing kit v1.1 (Applied Biosystems) followed by sequence analysis on a capillary ABI 3100 sequencer instrument (Applied Biosystems). 5′- and 3′-cDNA ends of each isolated desaturase cDNA were obtained using the RACE SMART™ Kit (Clontech, In vitro Sweden AB, Stockholm, Sweden). Gene-specific primers (Table S1) were designed at the extremity of the 5′- and 3′-untranslated regions (UTRs) and used for PCR amplification of full-length cDNA and subsequent verification of the integrity of the isolated desaturase cDNAs. All desaturase cDNA sequences have been deposited in the GenBank database under the accession numbers EU152399–EU152405.

2.4. Sequence and phylogenetic analyses

DNA sequences were analysed using the Sequencher V.3.0 software (Gene Codes Corporation, MI, USA), the Bioedit software (Version 5.0.9; Hall, 1999) and public non-redundant databases of both Blastn and Blastx searches (Altschul et al., 1997). Multiple sequence alignments were run using the Clustal W algorithm (Higgins et al., 1994) and edited in BOXSHADE (http://www.ch.embnet.org/index.html). Kyte and Doolitlle Hydropathy plots were obtained using Bioedit. Publicly available lepidopteran desaturase sequences were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov) and from the SilkDB genomic resource database (http://silkworm.genomics.org.cn).

The Neighbour-joining tree was built in MEGA v4 (JTT model, 1500 replicates, pariwise comparisons) as well as the calculations of evolutionary distances (Tamura et al., 2007).

2.5. Monitoring of desaturase expression levels by quantitative PCR

Total PG or FB RNAs were isolated from two biological samples of 10 *D. punctatus* females dissected 24 h before emergence (day −1) and at 0, 2 and 4 days post-emergence. cDNA sets from each pair of biological replicates were synthesized as described under Section 2.2 and balanced in equal concentrations. Twenty-five-microliter qPCR reactions were run on an Mx3000P v4.01 (Stratagene) using 20 ng cDNA as template with 200 nM GSPs (Table S2), 50 nM Rox Dye and the Platinum SYBR green qPCR SuperMix-UDG (Invitrogen). Primer sets were designed in the AlleleID software (PREMIER Biosoft International) with the following criteria: primers 18–23 base pairs in length, annealing temperature 60 ± 1 °C and a 75–125 nt amplicon. Each primer set was initially validated by calculating standard curves from serial dilutions of template cDNA (500 ng/μl to 0.4 ng/μl) and primers with amplification efficiencies (*E*) between 95 and 100% were used. qPCR amplification products from initial runs were checked on 2% agarose gels to verify the correct amplicon sizes and the absence of primer dimers. As a final validation, qPCR products were sequenced to ensure that the expected products were amplified. No-template controls were also run in parallel. Experiments with duplicated 25-μl reactions were replicated three times. Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 2 min, 45 cycles of 95 °C for 30s, 60 °C for 1 min, 72 °C for 1 min, followed by a dissociation curve analysis: 95 °C for 1 min, 60 °C for 30s and a gradual heating to 95 °C at 0.01 °C/s. Baseline cycle and threshold values were calculated automatically using default settings. Expression levels were normalized to the housekeeping gene (16S RNA) in the Mx3000 program (Stratagene) using the $(1 + E)^{-\Delta\Delta Ct}$ algorithm (Livak and Schmittgen, 2001). Log fold changes in expression within the gland were subjected to a one-way ANOVA. The groups were compared using the Ryan–Einot–Gabriel–Weilsh (REGW) procedure in the SPSS 16.0 package.

2.6. Heterologous expression in Saccharomyces cerevisiae

For the construction of yeast expression vectors containing the selected biosynthetic gene candidates, *i.e.*, either the *Dpu*-Δ11$_1$-APSQ, *Dpu*-Δ11$_2$-LPAE or *Dpu*-Δ9-KPSE genes, specific primers encompassing suitable restriction sites (Table S3) were designed for amplifying the open reading frames (ORFs) in combination with the Advantage® 2 PCR enzyme system (Clontech) and PG cDNA as template. PCR products were ligated and transformed as described and plasmid DNAs were purified using standard protocols. Subsequently, the cloned fragments were released by restriction enzyme digestion and agarose gel purified. Each linearized ORF was ligated into the pYEX-CHT vector (Patel et al., 2003). Prior to subcloning *Dpu*-Δ11$_2$-LPAE-ORF into the unique *EcoR1* site, the *EcoR1*-linearized pYEX-CHT vector was dephosphorylated in presence of calf intestine alkaline phosphatase (CIAP). After verification by sequencing the final DNA constructions, designated as pYEX-CHT vector only (control), pYEX-CHT-*Dpu*-Δ11$_1$-APSQ, pYEX-CHT-*Dpu*-Δ11$_2$-LPAE and pYEX-CHT-*Dpu*-Δ9-KPSE were each used for transformation into an elongase- (*elo1*) and desaturase-deficient (*ole1*) strain of *S. cerevisiae* (*MATa elo1*::*HIS3 ole1*::*LEU2 ade2 his3 leu2 ura3*) (Schneiter et al., 2000). For selection of uracil and leucine prototrophs, the transformed yeast was allowed to grow on the selective medium containing 0.7% YNB (w/o aa, with Ammonium sulfate) and a complete drop-out medium lacking uracil and leucine (ForMedium™ LTD, Norwich, England), 2% glucose, 1% tergitol (type Nonidet NP-40, Sigma–Aldrich Sweden AB, Stockholm, Sweden), 0.01% adenine (Sigma) and containing 0.5 mM oleic acid (Sigma) as extra fatty acid source. After 4 days at 30 °C, individual colonies were selected and inoculated in 10 ml selective medium at 30 °C and 300 rpm for 48 h. Yeast cultures were diluted to an OD_{600} of 0.4 in 10 ml fresh selective medium containing 2 mM $CuSO_4$ without or with supplementation with a biosynthetic intermediate, *i.e.*, *Z*9-16:Acid, *E*9-16:Me (Δ11-APSQ and Δ11-LPAE), *Z*8-14:Me (Δ11-APSQ), *Z*7-14:Me or *E*7-14:Me (Δ9-KPSE). All biosynthetic intermediates were prepared at a concentration of 500 mM in 96% Ethanol and added to reach a final concentration of 0.5 mM in the culture medium. After 48 h Cu^{2+}-induction, yeast cells were harvested and washed with sterile water two times. Yeast lipids were extracted from cell pellets at room temperature for 1 h in 500 μl chloroform:methanol (2:1, v:v) prior evaporation under a gentle stream of N_2 (Knipple et al., 1998; Moto et al., 2004). The dry yeast residues were subjected to base methanolysis to convert all fatty-acyl moieties into the corresponding methyl esters as described (Liénard et al., 2008) and recovered in *n*-hexane prior to GC–MS analysis. Dimethyl-disulfide (DMDS) adducts were prepared as described in Buser et al. (1983) in order to localize double bond positions in monoenes, whereas the 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) adducts were prepared as described in Marques et al. (2004) to localize double bond positions in conjugated dienes prior to GC–MS analysis.

2.7. GC–MS analyses

For the analysis of yeast fatty acid methyl esters (FAMEs) the GC (Hewlett Packard HP 5890II GC system) was coupled to a mass selective detector (MS) (HP 5972) and equipped with a polar INNOWax column (100% polyethylene glycol, 30 m × 0.25 mm × 0.25 μm, Agilent technologies). The GC–MS was operated in electron impact mode (70 eV) and the injector was configured in splitless mode at 240 °C with helium used as carrier gas (velocity: 30 cm/s). The oven temperature was maintained for 2 min at 70 °C and raised at a rate of 5 °C/min up to 225 °C, then increased at a rate of 25 °C/min up to 240 °C, then hold at 240 °C for 15 min.

DMDS and MTAD analyses were performed using the GC (HP 6890 GC system) equipped with a non-polar HP5-MS column (5%-Phenyl-methylpolysiloxane, 30 m × 0.25 mm × 0.25 μm, Agilent technologies) and coupled to a mass selective detector (HP 5973). The injector was configured in splitless mode and helium was used as carrier gas (velocity: 30 cm/s). For DMDS analyses, the oven temperature was maintained at 80 °C for 2 min followed by an increase at a rate of 10 °C/min to 180 °C, a rate of 3 °C/min to 260 °C and a final increase to 280 °C at a rate of 20 °C/min, held for 10 min. GC–MS analyses of MTAD adducts were performed as described in Marques et al. (2004).

2.8. FAME preparation

The *Z*9-16:Acid and *E*9-16:Me were purchased from Sigma and Larodan (Fine Chemicals AB, Sweden), respectively. The *E*7-14:Me was prepared from the corresponding *E*7-14:OH. The alcohol was dissolved in dimethylformamide (DMF) with pyridinium dichromate added as oxidant and the mixture was stirred overnight at room temperature. The acid product was extracted by diethyl ether and washed by distilled water. After drying over anhydrous sodium sulfate, the acid product was concentrated to dryness and used to prepare the methyl ester by acid-catalysed esterification. Briefly, the neat compound was dissolved in 0.5 M HCl/MeOH for 1 h at 80 °C. The reaction was subsequently neutralized by addition of 0.5 M KOH/MeOH and the methyl ester was recovered in hexane, washed with water and dried over anhydrous sodium sulfate.

The *Z*8-14:Me was synthesized by acid-catalysed esterification of its acid form.

The Δ9,Δ11-hexadecadienoate methyl esters (Δ9,Δ11-16:Me) used as standards were prepared from a mixture of the *Z*9,*E*11-hexadecadienol and the corresponding *E*9,*Z*11-, *Z*9,*Z*11- and *E*9,*E*11-isomers. In order to synthesize the corresponding methyl esters, the mixture was oxidized at 0 °C using Jones reagent (Berglund et al., 1993) followed by acid-catalysed esterification of the resulting acids. The *Z*7-14:Me was also synthesized following the Jones reagent's procedure prior to acid-catalysed esterification.

2.9. Intron pattern of Δ11-desaturases

Genomic DNA was isolated from abdominal tissue of a *D. punctatus* female according to the instructions provided in the DNeasy kit (Qiagen) and used as template in PCR reactions using gene-specific primers (Table S4) encompassing the presumed positions of the introns contained in Δ11-desaturase genes (Rosenfield et al., 2001). Specific PCR products were ligated, cloned and sequenced as described above. The non-intronic gDNA regions of each Δ11-desaturase were also amplified and sequenced to verify the absence of supplementary introns (GenBank accession numbers FJ466457–FJ466458). Orthologous genes from the SilkDB database were retrieved using the *Dpu* Δ11-desaturase sequences as query and analysed for intron positions.

3. Results

3.1. Screening for pheromone gland acyl-CoA desaturase cDNAs

D. punctatus PG tissue was screened using two distinct degenerated primer sets (Rosenfield et al., 2001; Roelofs et al., 2002) to amplify desaturase-encoding cDNA transcripts. In combination with RACE protocols, full-length cDNAs were obtained representing five putative desaturase genes that were designated as *Dpu*-Δ11$_1$-APSQ, *Dpu*-Δ11$_2$-LPAE, *Dpu*-Δ9-KPSE, *Dpu*-Δ9-NPVE and *Dpu*-Δ9-GATD respectively, in accordance with computational analyses and a desaturase-specific nomenclature (Knipple et al., 2002). All five primary proteins displayed high sequence similarities with distinct classes of insect desaturases, with whom they share common ancestry as indicated by a phylogenetic reconstruction (Fig. 1). One of these full-length cDNA transcripts, *Dpu*-Δ11$_1$-APSQ spanned 1204 bp and encompassed an ORF of 963 bp encoding a protein of 321 aa residues. A second full-length desaturase transcript, *Dpu*-Δ11$_2$-LPAE spanned 1670 bp and encoded an ORF of 1038 bp and 346 aa residues. Its deduced amino-acid sequence showed 53% sequence similarity to *Dpu*-Δ11$_1$-APSQ and both transcripts displayed close phylogenetic relationships to previously reported moth Δ11-desaturases (Fig. 1). The three other transcripts were highly similar to previously reported Δ9-desaturases. The *Dpu*-Δ9-KPSE protein was a 353-aa residue long polypeptide that showed high sequence similarity to the moth Δ9 $C_{16} > C_{18}$ group. The protein was encoded by three alternative transcripts that spanned 1695 bp, 1774 bp or 1837 bp, respectively and differed only in the 3′ UTR region due to distinct putative polyadenylation signals. The fourth desaturase transcript, *Dpu*-Δ9-NPVE spanned 1542 bp and encompassed an ORF of 1062 bp encoding a protein of 354 aa residues. Its deduced aa sequence displayed 64% similarity to *Dpu*-Δ9-KPSE and clustered with gene members of the Δ9 $C_{18} > C_{16}$ group (Fig. 1). The fifth transcript, *Dpu*-Δ9-GATD, spanned 1999 bp comprising a 1113-bp long ORF encoding a protein with 371 aa. The latter sequence displayed 52% similarity to *Dpu*-Δ9-KPSE, 49% to *Dpu*-Δ9-NPVE and 71% with an unusual desaturase (GenBank accession no. AAQ12887) that introduced Δ9-unsaturations in acyl chains ranging from C_{14} to C_{26} in *Choristoneura parallela* (Liu et al., 2004). All proteins also displayed the structural motifs of membrane-bound desaturase proteins, including three conserved histidine-rich regions and two long stretches of hydrophobic amino-acid residues presumably spanning the endoplasmic reticulum membrane (Stuckey et al., 1990; Shanklin et al., 1994; Tocher et al., 1998; Michaelson et al., 1998; Shanklin and Cahoon, 1998; Martin et al., 2002) and were therefore concluded as representing typical fatty-acyl-CoA desaturases.

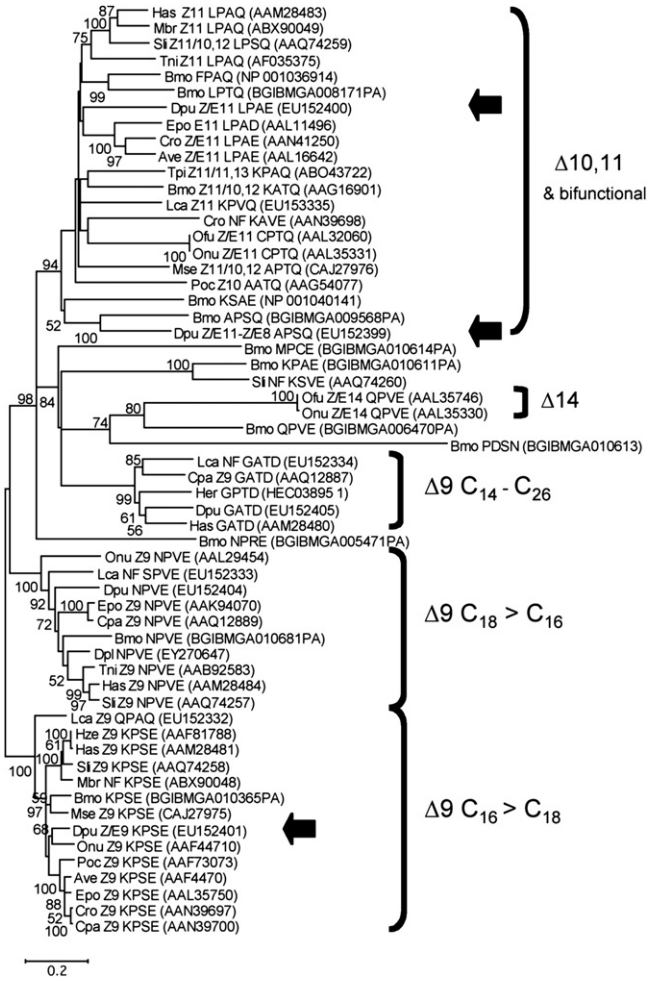

Fig. 1. Phylogeny of desaturase genes of lepidopteran insects. The Neighbour-joining tree was constructed using full-length aa sequences and the JTT algorithm (MEGA 4.0.). Numbers along branches indicate bootstrap support from 1500 replicates. Sequences are named according to the abbreviated species name, a desaturase catalytic activity (when assayed) and a four-amino-acid signature motif (SM) (Knipple et al., 2002). The accession numbers are indicated in parentheses. The desaturase genes involved in pheromone biosynthesis in *D. punctatus* (given the names Dpu *Z*/*E*11-LPAE, Dpu *Z*/*E*11-APSQ and Dpu *Z*/*E*9-KPSE) are indicated next to a black arrow. The tree was rooted on the Δ9-desaturase $C_{16} > C_{18}$ functional class. The abbreviated species names correspond to: Ave, *Argyrotaenia velutinana*; Bmo, *Bombyx mori*; Cpa, *Choristoneura parallela*; Cro, *Choristoneura rosaceana*; Dpl, *Danaus plexippus*; Dpu, *Dendrolimus punctatus*; Epo, *Epiphyas postvittana*; Has, *Helicoverpa assulta*; Her, *Heliconius erato*; Hze, *Helicoverpa zea*; Lca, *Lampronia capitella*; Mbr, *Mamestra brassicae*; Mse, *Manduca sexta*; Ofu, *Ostrinia furnacalis*; Onu, *Ostrinia nubilalis*; Poc, *Planotortrix octo*; Sli, *Spodoptera littoralis*; Tni, *Trichoplusia ni* and Tpi, *Thaumetopoea pityocampa*.

3.2. Monitoring of desaturase mRNA expression levels

Expression levels of all five transcripts were determined in PG and FB tissues of *D. punctatus* females at different ages by

quantitative real-time PCR (qPCR). The transcripts of the candidate biosynthetic desaturases *Dpu*-Δ11$_1$-APSQ and *Dpu*-Δ11$_2$-LPAE were expressed at high levels in the PG, with average log fold changes over age (LFC$_{all\text{-}days}$) of 5.4–11.4 compared to expression in the FB, respectively (Fig. 2A). In contrast, the transcripts for the three Δ9-desaturase candidates were found expressed at relatively similar levels in the PG and FB tissues, with differences in LFC$_{all\text{-}days}$ ranging on average from 1.06 to 2.23 (Fig. 2A). The relative expression of each desaturase transcript within the PG was determined relative to the housekeeping gene (Fig. 2B). This confirmed that *Dpu*-Δ11$_1$-APSQ and *Dpu*-Δ11$_2$-LPAE are highly expressed transcripts in the gland (LFC$_{all\text{-}days}$ 5.6 and 15.73, respectively). Although expressed similarly in both PG and FB over the life course of the insect, *Dpu*-Δ9-KPSE PG expression levels averaged those of *Dpu*-Δ11$_1$-APSQ (Fig. 2B). Interestingly, whereas the latter *Dpu*-Δ11$_1$-APSQ gene is significantly upregulated prior to eclosion (LFC$_{day\ 1}$ = 9.55), its expression decreased over time in mature females (LFC$_{day\ 4}$ = 4.39) and the opposite pattern was observed for *Dpu*-Δ9-KPSE (LFC$_{day\ 1}$ = 6.72 to LFC$_{day\ 4}$ = 10.35). As evidenced in other moth species (*e.g.*, Rosenfield et al., 2001; Jeong et al., 2003), the very low abundance of both *Dpu*-Δ9-NPVE and *Dpu*-Δ9-GATD transcripts in the PG suggested that they are unlikely to be involved in sex-pheromone biosynthesis. All together qPCR analysis demonstrated that both *Dpu*-Δ11$_1$-APSQ and *Dpu*-Δ11$_2$-LPAE transcripts are in addition to *Dpu*-Δ9-KPSE highly expressed in the gland, thereby representing the three most likely desaturase candidates associated with pheromone production in this species.

3.3. Heterologous expression in yeast

To investigate the enzyme activity and substrate specificity, the *Dpu*-Δ11$_1$-APSQ, *Dpu*-Δ11$_2$-LPAE or *Dpu*-Δ9-KPSE ORFs were subcloned into the copper-inducible pYEX-CHT expression vector (Patel et al., 2003) and transformed into the *elo1 ole1 S. cerevisiae* strain (Schneiter et al., 2000). DMDS analyses of FAMEs from Cu^{2+}-induced yeast transformed with the pYEX-CHT-only vector did not evidence the presence of unsaturated fatty acyls except from the supplemented *Z*9-18:Me (Fig. 3A). Cu^{2+}-induced yeast transformed with *Dpu*-Δ11$_1$-APSQ did not produced any biosynthetic Δ11-monoenes as evidenced by the absence of products at *m*/*z* 245 (Fig. 3B), in contrast to *Dpu*-Δ11$_2$-LPAE, which produced large amounts of Δ11-monoenoic acids, in particular the *Z*11-16:Me and *Z*11-18:Me in a 1.5:1 ratio (Fig. 3C). All *Dpu*-Δ11$_2$-LPAE DMDS adducts exhibited a diagnostic ion at *m*/*z* 245 in addition to the complementary ions at *m*/*z* 117 and 362 [M^+] for DMDS adducts of *Z*11-16:Me and ions at *m*/*z* 145 and 390 [M^+] for DMDS adducts of *Z*11-18:Me. The geometrical configuration of the *Z*11-18:Me was obtained by comparing its retention time with authentic synthetic standards' DMDS adducts from both *Z* and *E*11-18:Me. In addition, minor monoenes were detected whose DMDS adducts corresponded to the Δ11-12:Me (*m*/*z* 306 [M^+], 245 and 61), and to *Z* and *E*11-14:Me (*m*/*z* 334 [M^+], 245 and 89).

GC–MS analyses of FAMEs evidenced that *Dpu*-Δ9-KPSE produced a series of Δ9-monoenes. All its DMDS adducts exhibited the diagnostic ion at *m*/*z* 217 and were identified as *Z* and *E*9-12:Me

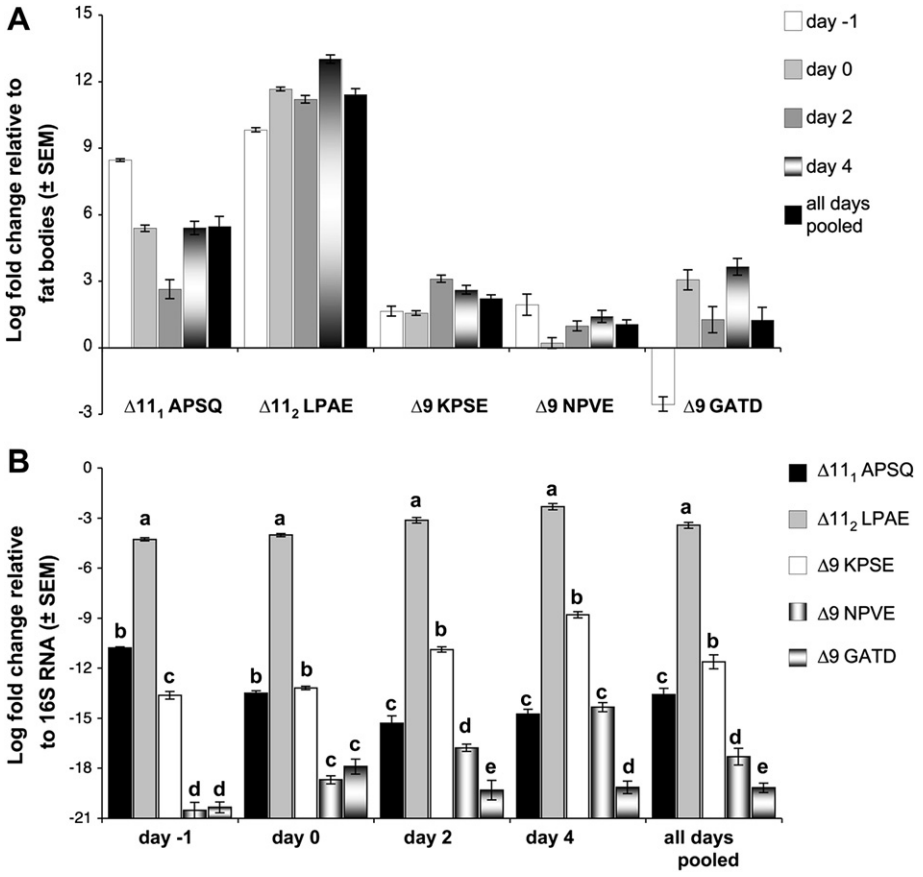

Fig. 2. Differential and temporal expression patterns of desaturase transcripts isolated from *D. punctatus* PG cDNA and monitored by qPCR. RNAs were extracted from the PG of females one day prior eclosion (day −1) and 0, 2 or 4 days post-emergence. The mean relative $\log_2$ fold change expression scores were calculated from raw cycle threshold (Ct) values (±SEM, $n = 6$). (A) Log fold change relative to the expression of the 16S RNA (normalizer) and calibrated to expression in FB. (B) Log fold change in PG expressed relative to the housekeeping gene (16S RNA); different letters indicate a significant difference at the 0.05 level in expression level between genes for a given age class (REGW post-hoc test).

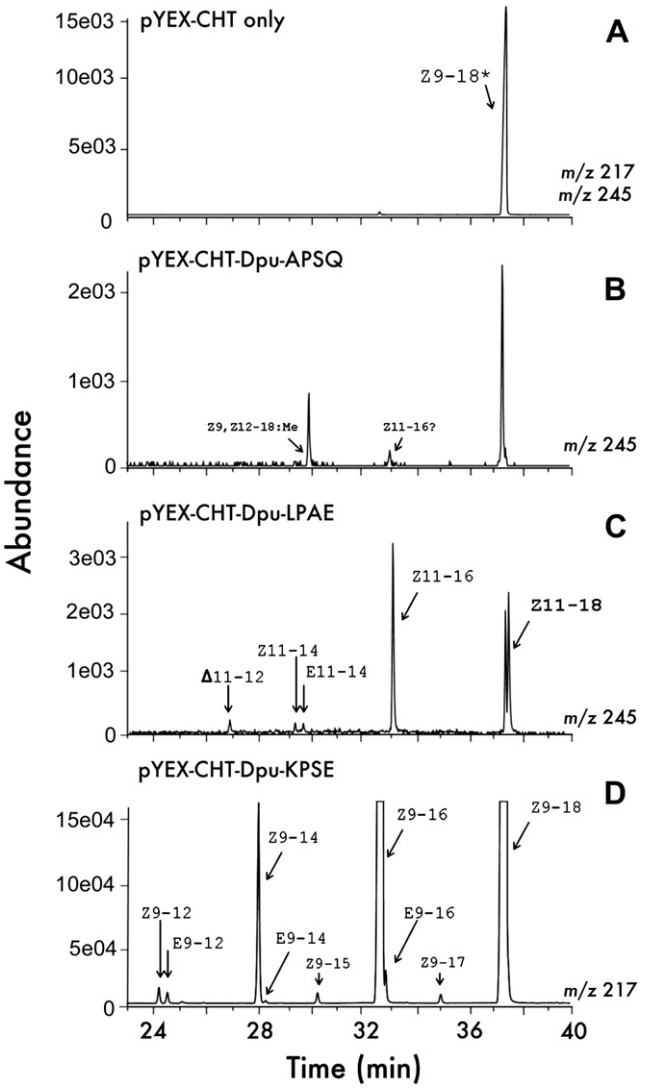

Fig. 3. GC–MS analysis of DMDS derivatives from methanolyzed Cu^{2+}-induced yeast extracts transformed with (A) control pYEX-CHT vector, (B) pYEX-CHT-*Dpu*-$\Delta11_1$-APSQ (C) pYEX-CHT-*Dpu*-$\Delta11_2$-LPAE and (D) pYEX-CHT-*Dpu*-Δ9-KPSE. The chromatogram traces represent the ion currents obtained by selection of the characteristic ion of Δ11-DMDS adducts at *m*/*z* 245 or Δ9-DMDS adducts at *m*/*z* 217. The asterisk (*) in (A) indicates the exogenous Z9-18:Me precursor provided to the deficient yeast prior to copper induction.

(*m*/*z* 306 [M^+], 217, 89), *Z* and *E*9-14:Me (*m*/*z* 334 [M^+], 217, 117), *Z* and *E*9-16:Me (*m*/*z* 362 [M^+], 217, 145) and *Z*9-18:Me (*m*/*z* 390 [M^+], 217, 173) (Fig. 3D). Interestingly, the *Dpu*-$\Delta11_1$-APSQ enzyme did produce a series of mono-unsaturated products whose DMDS adducts exhibited the characteristic ion at *m*/*z* 203. These mono-enes were identified as Δ8-12:Me, Δ8-14:Me and Δ8-16:Me (Fig. 4). In order to check whether the latter enzyme was able to use the *Z*8-14:Me as substrate to produce 7,9-14:dienoic acyl precursors, which could in turn lead to the 5,7-12:dienoic pheromone intermediates, we supplemented yeast transformed with *Dpu*-$\Delta11_1$-APSQ with the *Z*8-14:Me precursor. GC–MS analyses of yeast lipid extracts did not reveal the production of any C14 dienoic acids after supplementation (data not shown), which therefore excluded the second unsaturation step to occur through a 1,4-dehydrogenation mechanism.

When supplemented with the *Z*9-16:Acid, yeast cells transformed with *Dpu*-$\Delta11_1$-APSQ did not produce the Δ8-unsaturated FAMEs, but produced significant amount of di-unsaturated 9,11-C_{16} methyl esters (Fig. 5A, B), which exhibited an abundant molecular ion at *m*/*z* 266 (Fig. 5E). GC–MS analyses of MTAD derivatives confirmed these components to be 9,11-hexadecadienoates, which exhibited the characteristic ions at *m*/*z* 379 [M^+], *m*/*z* 322 and *m*/*z* 222 (base peak). The isomers were identified as the *Z*9,*E*11-16:Me and *E*9,*E*11-16:Me by comparing their retention times and mass spectra with those of the corresponding Δ9,Δ11-16:Me synthetic standards (Fig. 5D). When supplemented with *E*9-16:Me, yeast cells transformed with *Dpu*-$\Delta11_1$-APSQ produced all four 9,11-16:Me isomers. In contrast, when supplemented with the *Z*9-16:Me, yeast extracts of *Dpu*-$\Delta11_2$-LPAE contained no 9,11-16:Me (Fig. 5C), but contained small amounts of *E*9,*Z*11- and *E*9,*E*11-16:Me when supplemented with *E*9-16:Me (data not shown).

Another potential mono-unsaturated biosynthetic intermediate found in glands of *D. punctatus* is the *Z*7-14:acyl (Zhao et al., 2004). When supplementing *Z*7-14:Me to yeast transformed with the *Dpu*-Δ9-KPSE, the *Z*/*E*9-desaturase was able to catalyse the production of minor amounts of the *Z*7,*E*9-14:Acid (Fig. 6A, B), which exhibited an abundant molecular ion at *m*/*z* 238 (Fig. 6E). When supplemented with the *E*7-14:Me, the enzyme produced both *E*7,*Z*9-14:Acid and *E*7,*E*9-14:Acid (Fig. 6C, D). The Δ7,Δ9 double bond positions were confirmed by MTAD derivatization (Fig. 6F) and the geometrical configurations were rationalized by comparison with the elution order of Δ9,Δ11-16:Me.

Overall functional expression conclusively demonstrated that the three *Dpu*-$\Delta11_1$-APSQ, *Dpu*-$\Delta11_2$-LPAE and *Dpu*-Δ9-KPSE encode functional enzymes exhibiting a combination of specific desaturase activities consistent with the biosynthesis of all main sex-pheromone fatty-acyl precursors in *D. punctatus*.

3.4. Acyl-CoA Δ11-desaturases: divergence in primary structures and intron patterns

When reconstructing the phylogeny of Lepidopteran desaturases, both *Dpu*-$\Delta11_1$-APSQ and *Dpu*-$\Delta11_2$-LPAE transcripts appeared to represent two duplicate genes of the Δ11-desaturase subfamily (Fig. 1). Their primary structure possessed all structural features of typical eukaryotic membrane-spanning desaturases including the four hydrophobic transmembrane regions and the three hydrophilic HIS-containing motifs as illustrated in Fig. 7A, B (Stuckey et al., 1990; Shanklin et al., 1994; Knipple et al., 1998; Rosenfield et al., 2001; Jeong et al., 2003). The two polypeptide chains are overall relatively conserved (53%, uncorrected *p*-distance = 0.444) and both are functional desaturase proteins notwithstanding that they evolved distinct substrate specificities (Figs. 3–5). The proportion of sites at which the two genes differ is higher in the transmembrane regions (TM1–TM4) (uncorrected *p*-distance = 0.551) than in their cytoplasmic regions (uncorrected *p*-distance = 0.407) (Fig. 7A, B), which suggests that non-synonymous substitutions may be afforded in the TM regions as long as the overall hydrophobic structure is maintained. In contrast, there should be more selective pressure operating on certain cytoplasmic domains that have been predicted to form the active channel interacting with fatty acid substrates (Sperling et al., 2003).

Dpu-$\Delta11_1$-APSQ and *Dpu*-$\Delta11_2$-LPAE gene structures were characterized and compared with Δ11-homologous genes from *B. mori*. Four Δ11-homologous sequences were found in the silkworm database in addition to the gene corresponding to the unique Δ11-10,12-KATQ desaturase implied in *B. mori* pheromone biosynthesis (Moto et al., 2004). Both *D. punctatus* Δ11-desaturases were evidenced to have a close ortholog in *B. mori*, *i.e.*, *Dpu*-$\Delta11_1$-APSQ corresponded to *Bmo*-APSQ whereas *Dpu*-$\Delta11_2$-LPAE was most similar in sequence with the functional *Bmo*-KATQ (Fig. 1). Both *D. punctatus* Δ11 genes thus likely evolved following

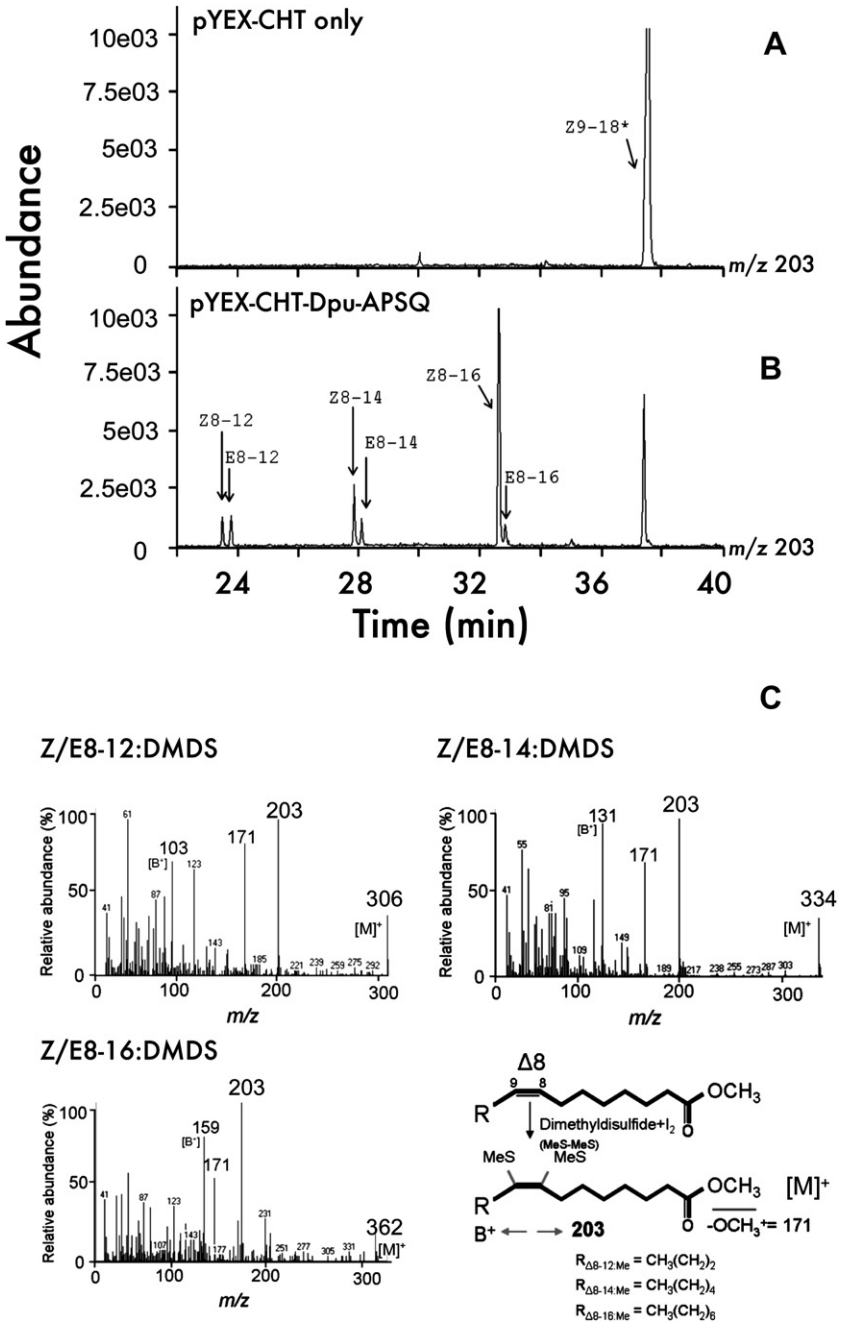

Fig. 4. GC–MS analysis of Δ8-mono-unsaturated intermediates produced by functional expression of the *Dpu*-Δ11$_1$-APSQ desaturase gene. Chromatogram traces represent DMDS adducts of methanolyzed yeast extracts of (A) the control pYEX-CHT vector and (B) the pYEX-CHT-*Dpu*-Δ11$_1$-APSQ and were obtained by selection of the characteristic ion of Δ8-DMDS adducts at *m*/*z* 203. The asterisk (*) refers to the exogenous *Z*9-18:Me precursor. (C) Mass spectra and molecular fragmentation of DMDS adducts of Δ8-12:Me, Δ8-14:Me and Δ8-16:Me.

a relatively ancient duplication event that took place prior to the divergence of the Bombycidae and Lasiocampidae families.

While lepidopteran Δ9-desaturases have been shown to display a three-intron gene structure (*i.e.*, Introns I–III) (Fig. 7C), Δ11-desaturase genes usually lack intron II (Rosenfield et al., 2001; Knipple et al., 2002), which has presumably been lost subsequent to the duplication between Δ9- and Δ11-desaturases. Accordingly, all predicted *B. mori* Δ11-desaturase genes as well as the *Dpu*-Δ11$_2$-LPAE were found to display a typical two-intron (I and III) pattern (Fig. 7C). Two variants of the *Dpu*-Δ11$_1$-APSQ Intron III presumably representing the two allelic versions of the gene were cloned that were identical except by a 224-bp long supplementary portion.

Although Intron I is typically conserved among desaturase genes in moths but also flies (Dallerac et al., 2000), several sets of primers targeting the boundaries of the expected *Dpu*-Δ11$_1$-APSQ intron I led to a PCR amplicon matching the size of its corresponding cDNA, which suggested that *Dpu*-Δ11$_1$-APSQ may have lost this intron.

4. Discussion

Pheromone biosynthetic desaturases with specific substrate affinities, chain-length preferences and *Z* or *E* stereospecificities contribute significantly to the diversity of chemicals used as moth mating signals (*e.g.*, Bjostad and Roelofs, 1984; Tillman et al., 1999;

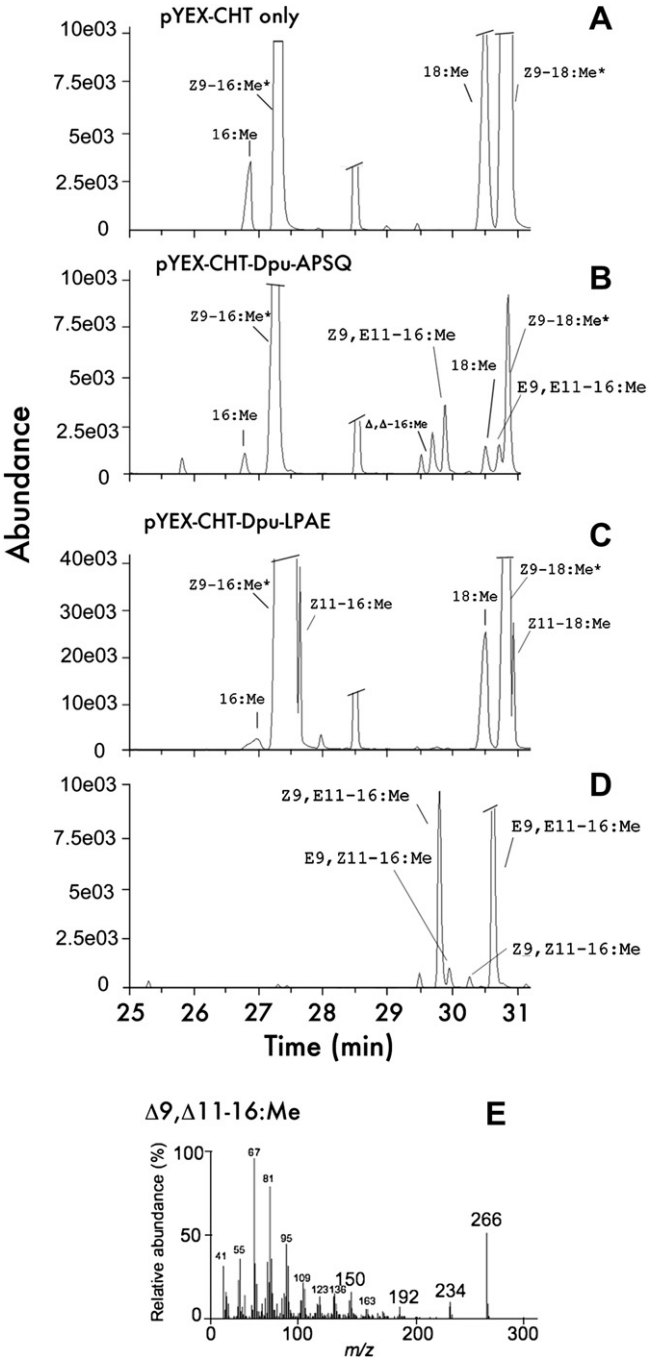

Fig. 5. GC–MS analysis of conjugated fatty acid intermediates from methanolyzed Cu^{2+}-induced yeast transformed with *D. punctatus* Δ11-desaturase genes and supplemented with *Z*9-16:Acid. FAME extracts from yeasts transformed with (A) pYEX-CHT only, (B) pYEX-CHT-*Dpu*-$\Delta11_1$-APSQ and (C) pYEX-CHT-*Dpu*-$\Delta11_2$-LPAE. The chromatogram traces are obtained by selection of the characteristic ions of methyl hexadecadienoates at *m*/*z* 266, 234, 192 and 150. Asterisks (*) refer to the exogenous *Z*9-18:Me and the biosynthetic intermediate *Z*9-16:Acid. (D) GC–MS chromatogram of the isomers of 9,11-16:Me synthetic standards. (E) Mass spectrum of the *Z*9,*E*11-16:Me.

Knipple et al., 1998, 2002; Rosenfield et al., 2001; Liu et al., 1999, 2002a,b, 2004; Hao et al., 2002; Roelofs et al., 2002; Jeong et al., 2003; Moto et al., 2004; Matoušková et al., 2007; Serra et al., 2006, 2007, Liénard et al., 2008). We demonstrated that the biosynthesis of pheromone components used in *D. punctatus* is likely the result of the combined action of three distinct desaturases (Fig. 8). Phylogenetic analysis indicated that the three desaturase transcripts, namely *Dpu*-$\Delta11_1$-APSQ, *Dpu*-$\Delta11_2$-LPAE and *Dpu*-Δ9-KPSE are related to moth desaturases belonging to the Δ11- or the Δ9 $C_{16} > C_{18}$ subfamilies, respectively (Fig. 1) and are expressed at high levels in the PG tissue (Fig. 2). *In silico* and tissue distribution approaches are however not conclusive without the final demonstration that the transcripts encode functional proteins with activities consistent with pheromone production in the species investigated. Cloning of each candidate desaturase ORF in the pYEX-CHT vector and subsequent heterologous expression in *S. cerevisiae* conclusively evidenced that the PG transcript with highest expression, *Dpu*-$\Delta11_2$-LPAE (Fig. 2) produces the biosynthetic intermediate *Z*11-18:acyl (Fig. 3), which is in accordance with an earlier *in vivo* biochemical study (Zhao et al., 2004). Deuterium labelling previously showed that this precursor is subsequently chain-shortened to the *Z*5-12:acyl precursor by three cycles of β-oxidation and finally reduced to form the *Z*5-12:OH (Zhao et al., 2004).

The pathway leading to the *Z*5,*E*7-12:OH could not be tackled in the earlier *in vivo* studies and we demonstrate that among moths for which functional studies of pheromone biosynthesis are available to date, *D. punctatus* is a complex case (Fig. 8). *In vivo* labelling showed that the *Z*11-18:acyl leads to the *Z*9-16:acyl through chain-shortening (Zhao et al., 2004) and we here conclusively demonstrate that the *Z*9-16:acyl is then used as substrate by a second Δ11-enzyme, *Dpu*-$\Delta11_1$-APSQ that catalyses an *E*11-desaturation reaction to form the *Z*9,*E*11-16:acyl precursor (Fig. 5). We also show that *Dpu*-Δ9-KPSE can introduce an *E*9-desaturation in the chain-shortened *Z*7-14:acyl to produce the *Z*7,*E*9-14:acyl (Fig. 6). Both *Z*9, *E*11-16 and *Z*7,*E*9-14:acyls can in turn lead to the *Z*5,*E*7-12:acyl through β-oxidation and be reduced to the corresponding alcohol. In *Dendrolimus* spp. females, the peak of calling activity is typically observed between day 1 and day 2 following eclosion (Zhao et al., 2004; Rotundo et al., 2004; Kong et al., 2007), which coincides with the time-window of expression of the three active desaturases. Whereas *Dpu*-$\Delta11_1$-APSQ is upregulated before eclosion, the gene expression noticeably decreases after emergence (Fig. 2), which interestingly coincides with the upregulation of the *Dpu*-Δ9-KPSE. All together *Dpu*-$\Delta11_2$-LPAE leads to the production of the *Z*11-18:acyl whereas both *Dpu*-$\Delta11_1$-APSQ and *Dpu*-Δ9-KPSE provide two alternative routes towards key di-unsaturated intermediate precursors of the female sex pheromone of *D. punctatus* (Fig. 8). Future work involving *in vivo* knockdown studies could be attempted to decipher if either enzyme is prevalent in forming the *Z*5,*E*7-12:acyl precursor. The *RNAi* approach has generated satisfactory data in the P50 strain of *B. mori* (Ohnishi et al., 2006) but it however failed in other strains of *B. mori* as well as in other moth species (Matsumoto S., personal communication).

Similarly to *Dpu*-Δ9-KPSE, the involvement of a moth Δ9 $C_{16} > C_{18}$ desaturase in the production of a di-unsaturated pheromone precursor was demonstrated in *S. littoralis* (Rodríguez et al., 2004) but remains a rare circumstance in moth pheromone biosynthesis. Hence, moth pheromone desaturases of the Δ9 $C_{16} > C_{18}$ functional class are usually restricted to the production of *Z*9-monoenes of C_{16} and C_{18} plus minor amounts of C_{14}:Δ9 (Rosenfield et al., 2001; Jeong et al., 2003; Liu et al., 2004). Orthologous dipteran Δ9 $C_{16} > C_{18}$ desaturases have been shown to serve a role in the production of cuticular pheromones in male and female flies (Coyne et al., 1999; Dallerac et al., 2000; Ferveur, 2005). Whereas the *Drosophila melanogaster desat1* displays a typical $C_{16} > C_{18}$ substrate preference, its paralog *desat2* has evolved a high affinity for myristic acid and produces only minor amounts of C_{16}:Δ9 and C_{18}:Δ9 (Dallerac et al., 2000). This suggests that the substrate preference of contemporary moth Δ9-desaturases may also be highly derived from their ancestral activity and it is conceivable that they may have evolved unusual activities such as observed in

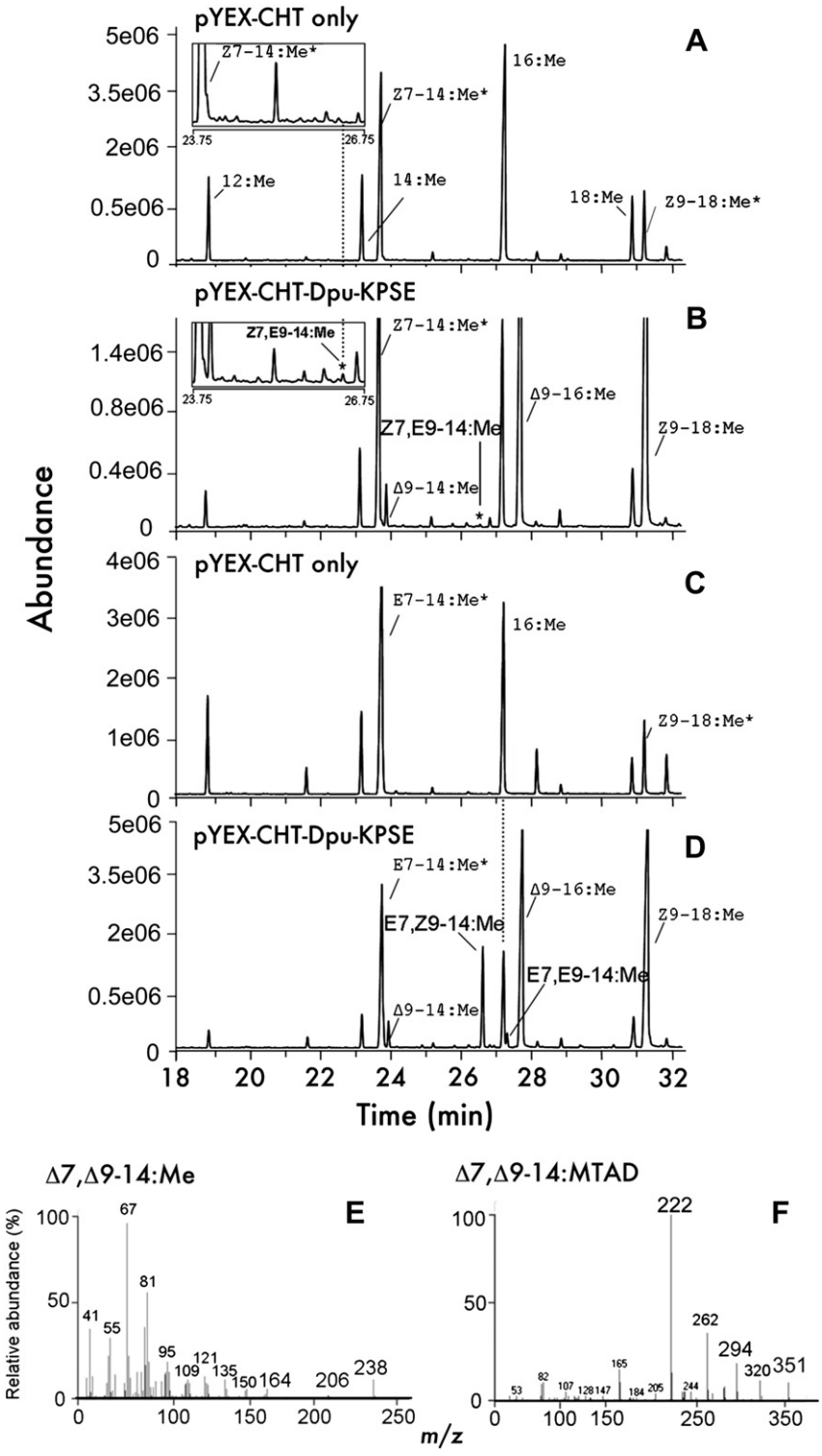

Fig. 6. GC–MS analysis of conjugated fatty acid intermediates from methanolyzed Cu^{2+}-induced yeast transformed with *Dpu*-Δ9-KPSE. FAME extracts from yeasts transformed with (A and C) pYEX-CHT only, (B and D) pYEX-CHT-*Dpu*-Δ9-KPSE supplemented with either *Z* or *E*7-14:Me. The chromatogram traces represent the total ion current (TIC) chromatograms. Asterisks (*) refer to the exogenous *Z*9-18:Me and the biosynthetic intermediate *Z*7-14:Me or *E*7-14:Me. (E) Mass spectrum of the *Z*7,*E*9-14:Me. (F) Mass spectrum of Δ7, Δ9-14:Me MTAD derivatives.

D. punctatus. Desaturases involved in the formation of UFAs have been characterized in other insect lineages and include a Δ9-desaturase (C_{18}:Δ9 > C_{16}:Δ9) from the house cricket *Acheta domesticus* (Orthoptera) (Riddervold et al., 2002) and from the white-tailed bumblebee *Bombus lucorum* (Hymenoptera) (Matoušková et al., 2008) as well as a Δ12-desaturase involved in the production of linoleic acid in *A. domesticus* and the flour beetle, *Tribolium castaneum* (Coleoptera) (Zhou et al., 2008).

All species in the Lasiocampidae family for which a sex pheromone has been identified to date use derivatives of the *Z*5-12 and

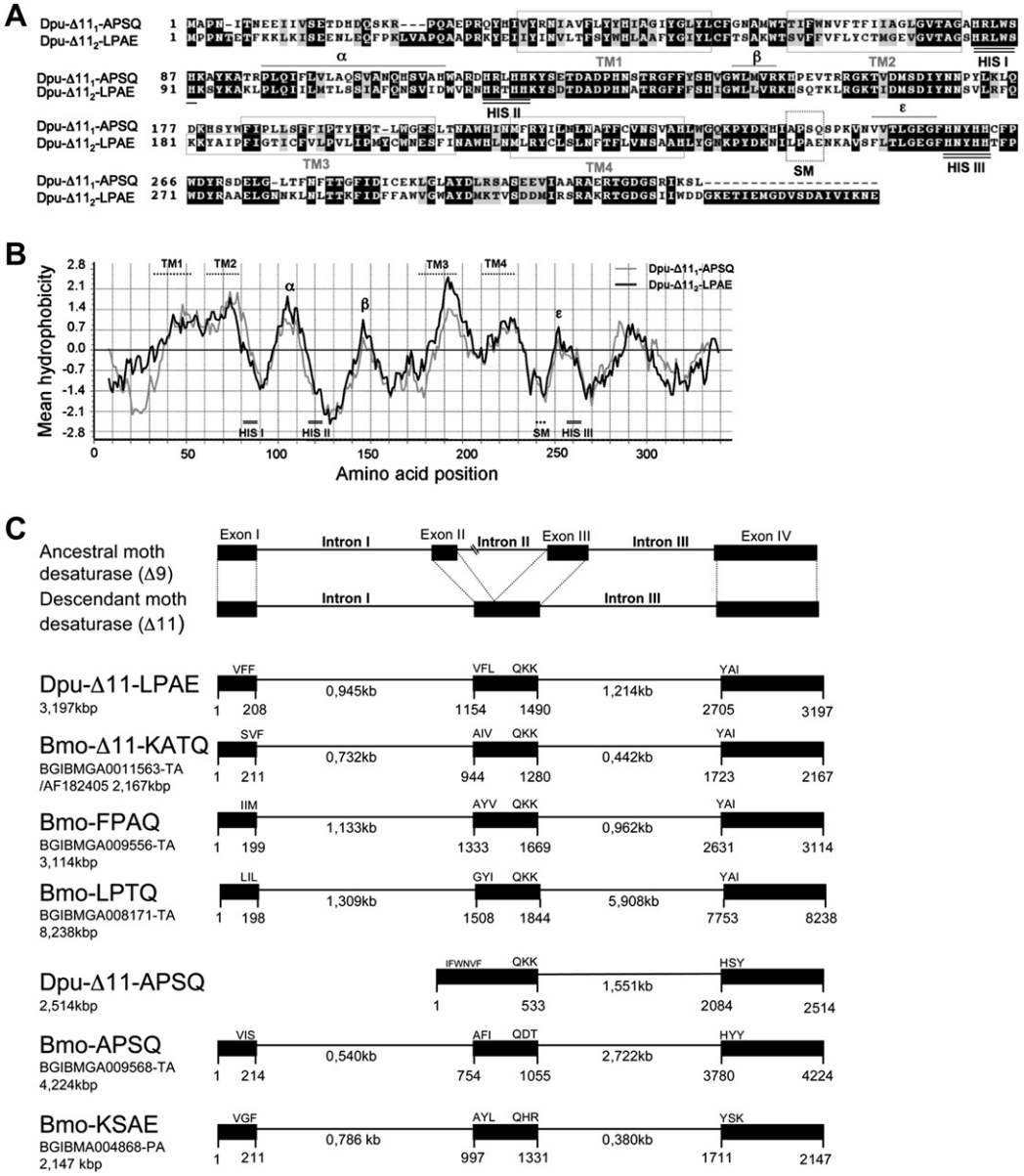

Fig. 7. Amino-acid sequence alignment and intron patterns of the two functional acyl-CoA Δ11-desaturases from *D. punctatus*. (A) Black, grey and white backgrounds indicate aa identities, conservative and non-conservative substitutions, respectively. Boxed regions indicate the hydrophobic transmembrane domains (TM1–TM4) and the four-amino-acid signature motif (SM). HIS refers to Histidine-rich domains and α, β and ε represent hydrophobic motifs (Knipple et al., 2002). (B) Hydropathy plots of the *Dpu*-Δ11$_1$-APSQ and *Dpu*-Δ11$_2$-LPAE proteins. (C) Δ11-Desaturase genes from *D. punctatus* and *B. mori*. Intron positions are located between the last amino acid specified by the three codons on the 5′ side of a splice junction and the first amino acid on the 3′ side of a splice junction. Intron positions are conserved among all Δ11-desaturase genes, except in *Dpu*-Δ11$_1$-APSQ. The *B. mori* functional Δ11,Δ10/12-desaturase is referred to as Bmo-Δ11-KATQ. (k)bp, (kilo)base pair.

Δ5,Δ7-12:acyls. This includes species of the *Dendrolimus* genus (*e.g.*, Zhao et al., 1993; Klun et al., 2000; Kong et al., 2007 and references therein), the *Malacosoma* genus (Kochansky et al., 1996; Schmidt et al., 2003; Rotundo et al., 2004) and the *Gastropacha* genus (Bestmann et al., 1993). Since closely related species share common evolutionary histories, common pheromone components likely derive from the sharing of similar biosynthetic pathways (reviewed in Cardé and Haynes, 2004). Placed in a phylogenetic context, we rationalize that other Lasiocampidae species may use orthologous desaturases to produce their pheromone precursors. For instance, GC–MS analyses of yeast lipid extracts indicated that the *Dpu*-Δ9-KPSE is capable of producing *E*9-16:acyl (Fig. 5) and when supplemented with the latter substrate *Dpu*-Δ11$_1$-APSQ forms the *E*9,*Z*11-16:acyl. Similarly, *Dpu*-Δ9-KPSE exhibits a significant activity on the chain-shortened *E*7-14:acyl that leads to *E*7,*Z*9-14:acyl (Fig. 6). Orthologous desaturases of other lasiocampids could also produce *E,Z* isomers, which would lead to the main *E*5,*Z*7-12:acyl precursor in *Dendrolimus houi*, providing that the appropriate number of β-oxidation cycles subsequently occur in the gland (Zhao et al., 1993; Kong et al., 2001, 2007) but also in *Malacosoma neustrium* and *Malacosoma americanum* (Kochansky et al., 1996; Rotundo et al., 2004). The stereochemical mechanism catalysing the formation of *E,E*-isomers in experiments where *Z*-precursors were supplemented (and *vice versa*) remains to be investigated. Similarly to *Dpu*-Δ11$_1$-APSQ, the *Z*5,*Z*7-12:Ald used in *Malacosoma disstria* (Schmidt et al., 2003) could be produced from an orthologous Δ11$_1$-APSQ desaturase that would also produce *Z,Z* isomers.

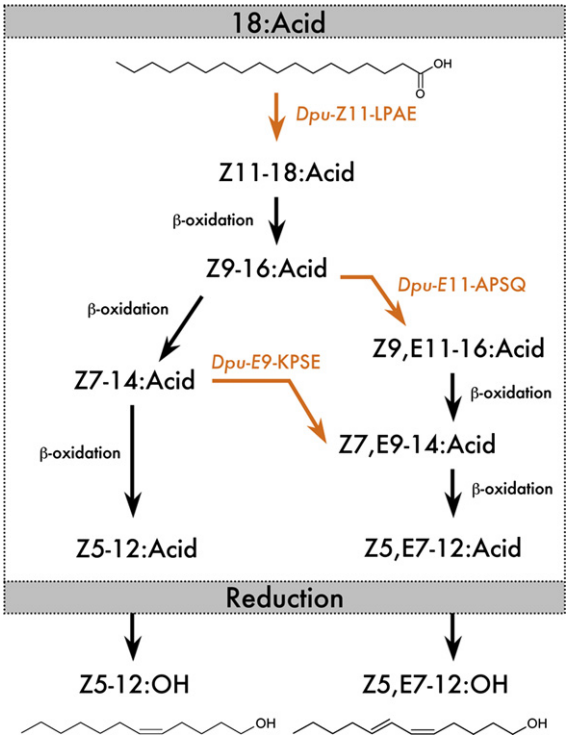

Fig. 8. Proposed biosynthetic pathway towards the female sex pheromone of *D. punctatus*. *Dpu*-Δ11$_2$-LPAE leads to the production of the *Z*11-18:Acid whereas both *Dpu*-Δ11$_1$-APSQ and *Dpu*-Δ9-KPSE are involved in the introduction of *E* double bonds that form key di-unsaturated intermediate pheromone precursors.

Phylogenetic analysis indicates that *Dpu*-Δ11$_1$-APSQ belongs to the Lepidoptera-specific Δ11-desaturase lineage but in addition to the expected Δ11-desaturase activity it also displays an unusual Δ8-desaturase activity. In contrast to *Dendrolimus superans sibiricus* where the minor *E*6-12:OH/Ald pheromone components (Klun et al., 2000) are likely the result of a similar Δ8-activity followed by β-oxidation and subsequent reduction/oxidation, no Δ8-monoenes were detected in the gland in *D. punctatus* (Zhao et al., 2004). *In vitro*, no Δ8 UFAs were observed when the enzyme was supplemented with *Z*9-16:acyl but in this latter case, the enzyme catalysed the production of 9,11-16:dienes. These observations could be explained by a down-regulation or inhibition of the Δ8-activity in presence of *Z*9-16:acyl. A similar mechanism has been postulated in *S. littoralis* where a Δ9-desaturase could produce the *Z*9-14:Acid from 14:Acid but not when supplemented with *E*11-14:Acid, in which case it instead produced a 9,11-14:diene (Rodríguez et al., 2004).

Our results also demonstrate the involvement of two different Δ11-desaturases involved in pheromone production in one moth species. Extensive gene duplications took place in the course of evolution of the Δ11-subfamily as supported by multiple Δ11-desaturase gene copies in *B. mori* (Fig. 7), *C. pomonella* (Knipple et al., 2002), *Choristoneura* spp. (Hao et al., 2002; Liu et al., 2004), *M. sexta* (Matoušková et al., 2007) and *Plodia interpunctella* (Tsfadia et al., 2008). Classical evolutionary models predict that duplicated genes may be lost over time or remain in the genome as pseudogenes, whereas when providing an evolutionary advantage they may evolve new functions (neofunctionalization) or partition the ancestral gene's functions (subfunctionalization) (Hughes, 1994; Lynch and Conery, 2000; Lynch and Force, 2000; Otto and Yong, 2002). Moth desaturases have also been predicted to evolve under a birth-and-death evolutionary pattern (Roelofs and Rooney, 2003; Nei and Rooney, 2005). All together, this supports that the two *D. punctatus* Δ11-desaturase genes represent a novel case of evolution by subfunctionalization in the moth pheromone biosynthetic machinery.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ibmb.2010.04.003.

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