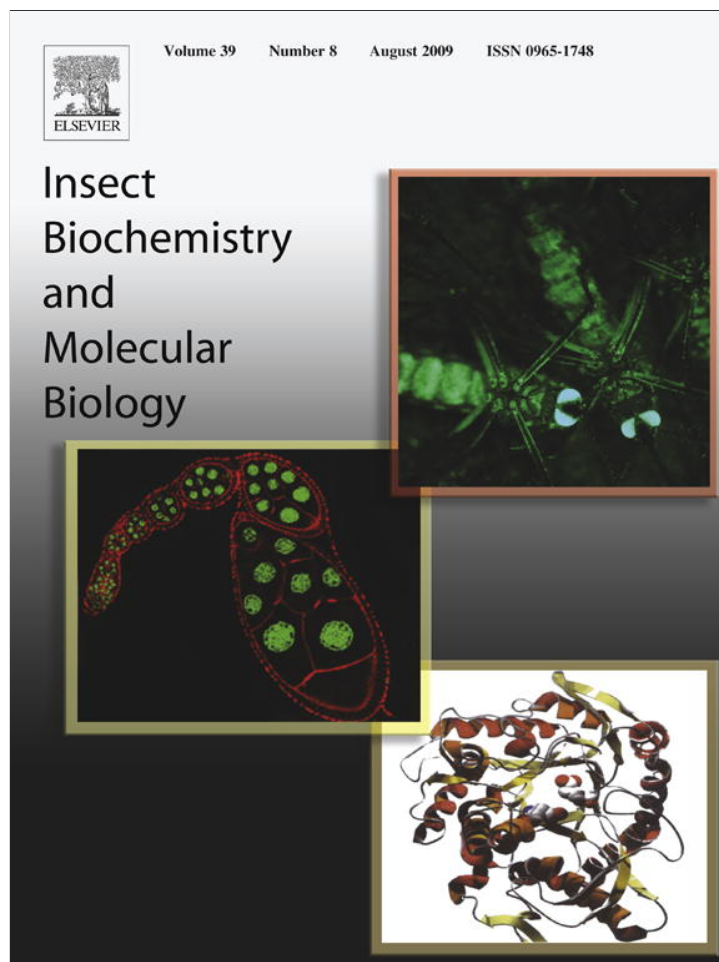


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QTL analysis of sex pheromone blend differences between two closely related moths: Insights into divergence in biosynthetic pathways

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ABSTRACT

To understand the evolution of premating signals in moths, it is important to know the genetic basis of these signals. We conducted Quantitative Trait Locus (QTL) analysis by hybridizing two noctuid moth species, *Heliothis virescens* (Hv) and *Heliothis subflexa* (Hs), and backcrossing the F₁ females to males of both parental species. One of these backcrosses (F₁ × Hs) was a biological replicate of our previous study (Sheek et al., 2006) and served to test the robustness of our previous findings. The backcross to Hv was designed to reveal QTL with recessive inheritance of the Hv character state. This study confirms previously discovered QTL, but also reports new QTL. Most importantly, we found relatively large QTL affecting Z9-16:Ald, the critical sex pheromone component of Hs. For Z9-14:Ald, the critical sex pheromone component of Hv, as well as for the minor pheromone compound 14:Ald, we found QTL in which the change in pheromone ratio was opposite-to-expected. Linking QTL to the biosynthetic pathways of the pheromone compounds of Hv and Hs implicates several candidate genes in the divergence of these premating signals, the most important of which are acetyl transferase, one or more desaturase(s), and a fatty acyl reductase or alcohol oxidase.

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1. Introduction

Moths rely on production and perception of specific blends of volatile compounds to find mating partners, and genetic divergence in this aspect of moth sexual communication is considered to be an important component in moth speciation (e.g., Roelofs et al., 2002; Coyne and Orr, 2004). From a population genetics perspective, however, the evolutionary diversification in moth pheromone-based communication systems is difficult to understand, because these and other premating signals are generally expected to be under stabilizing selection (Gerhard, 1991; Löfstedt, 1993; Linn and Roelofs, 1995; Butlin, 1995; Butlin and Trickett, 1997; Phelan, 1997; Shaw and Parsons, 2002; Bürger et al., 2006), and stabilizing selection constrains evolutionary diversification (e.g., Coyne et al., 1997; Butlin and Trickett, 1997; Phelan, 1997). Unless stochastic events (Wade and Goodnight, 1998), pleiotropy, or some

environmental factor(s) causes directional selection strong enough to overcome the stabilizing selection (Raffa and Dahlen, 1995; Groot et al., 2006), evolutionary stasis is predicted. The balance between stabilizing selection and these other evolutionary forces is likely to be influenced by a) the number of genes involved in the initial divergence, b) the magnitude of effect of each gene on fitness-related phenotypes, and c) allelic interactions affecting fitness-related phenotypes (Coyne and Orr, 2004; Wade and Goodnight, 1998; Dieckmann and Doebeli, 1999; Kondrashov and Kondrashov, 1999; Whitlock and Phillips, 2000). Thus, understanding the genetic basis for sex pheromone differences between closely related species is essential for understanding the evolution of premating signals. Furthermore, linking quantitative and qualitative differences between species to specific genes could enable direct tests of selection on these genes.

Most studies on the genetics of pheromonal differences in moths have focused on changes in pheromone blends that can be explained by alteration in one or two enzymes, or by the sequence of chain-shortening and desaturation events (e.g., Löfstedt, 1993; Cossé et al., 1995; Haynes, 1997; LaForest et al., 1997; Roelofs and Rooney, 2003; Dopman et al., 2004; Tabata and Ishikawa, 2005). The differences between the multi-component pheromone blends of females of many moth species, such as *Heliothis virescens* (Hv) and *Heliothis subflexa* (Hs), cannot be explained by one enzyme (Jurenka,

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Table 1
Sex pheromone compounds of Hv and Hs.

	Hv ^a	Hs ^b
Z7-16:OAc	–	+
Z7-16:Ald	?	?
Z11-16:OAc	–	+
Z11-16:OH	+/-	++
Z11-16:Ald	+++	+++
Z9-16:OAc	?	?
Z9-16:Ald	?	++
16:Ald	?	?
Z9-14:Ald	++	?
14:Ald	?	?

+++ : major sex pheromone component; ++ : critical secondary sex pheromone component; + : secondary sex pheromone component; +/- : attractive at low amounts, repellent at higher amounts (Ramaswamy et al., 1985); ? : relative importance in the attraction of conspecific males or repulsion of heterospecific males has not been systematically tested so far, but seems to be relatively unimportant.

^a Summarized from Roelofs et al. (1974), Tumlinson et al. (1975, 1982), Klun et al. (1979, 1980), Vetter and Baker (1983), Ramaswamy et al. (1985), Teal et al. (1986), and Vickers and Baker (1997).

^b Summarized from Teal et al. (1981), Klun et al. (1982), Heath et al. (1990), and Groot et al. (2007).

2004), because compounds with three different functional groups (alcohols, aldehydes, and acetate esters) and two different chain lengths (C14 and C16) are involved (see Table 1 and Fig. 1). Since Hv and Hs hybridize in the laboratory, they have served as a model system for elucidating the genetic basis of the production of multi-component sex pheromone blends by females (Teal and Oostendorp, 1995; Teal and Tumlinson, 1997; Groot et al., 2004; Sheck et al., 2006) and male behavioral response (Vickers, 2006a,b; Baker

et al., 2006). For a more detailed description and visualization of our mating design and QTL analysis, see Groot et al. (2004).

By backcrossing F₁ hybrid females to the parental Hs we previously found five QTL affecting the proportions of seven sex pheromone components, each explaining 7–34% of the variance among the backcross offspring (Sheck et al., 2006; see Fig. 1). The largest QTL affected the relative amount of 14:Ald, which appears to be unimportant in attraction/repulsion of males of either species (Groot et al., unpubl. res). Three acetate ester components, produced only by Hs and not by Hv females, have a dual function: they increase attraction of conspecific Hs males but also inhibit attraction of heterospecific Hv males (Vickers and Baker, 1997; Vickers, 2002; Groot et al., 2006) and may thus have arisen due to positive selection.

Previously, we found two QTL for these acetates (Sheck et al., 2006). In contrast, for Z9-14:Ald, a critical pheromone component of Hv, we found a relatively small QTL, explaining only 9% of the variance. This component is essential, because without it Hv males are not attracted (Roelofs et al., 1974; Klun et al., 1979, 1980; Tumlinson et al., 1975, 1982; Pope et al., 1982; Vetter and Baker, 1983). We did not find any QTL for Z9-16:Ald, a critical pheromone component of Hs. Because sample sizes were limited in our previous study—the analysis was based on two families consisting of 45 females each, and the backcrosses were only carried out in one direction—we could not conclude that we had found all of the biologically important QTL. The current study was therefore designed to replicate and extend the Sheck et al. (2006) study. This study confirms three QTL that we reported earlier, two QTL that are likely the same, and seven new QTL. In addition, we found relatively large QTL affecting the relative amount of Z9-16:Ald, a critical

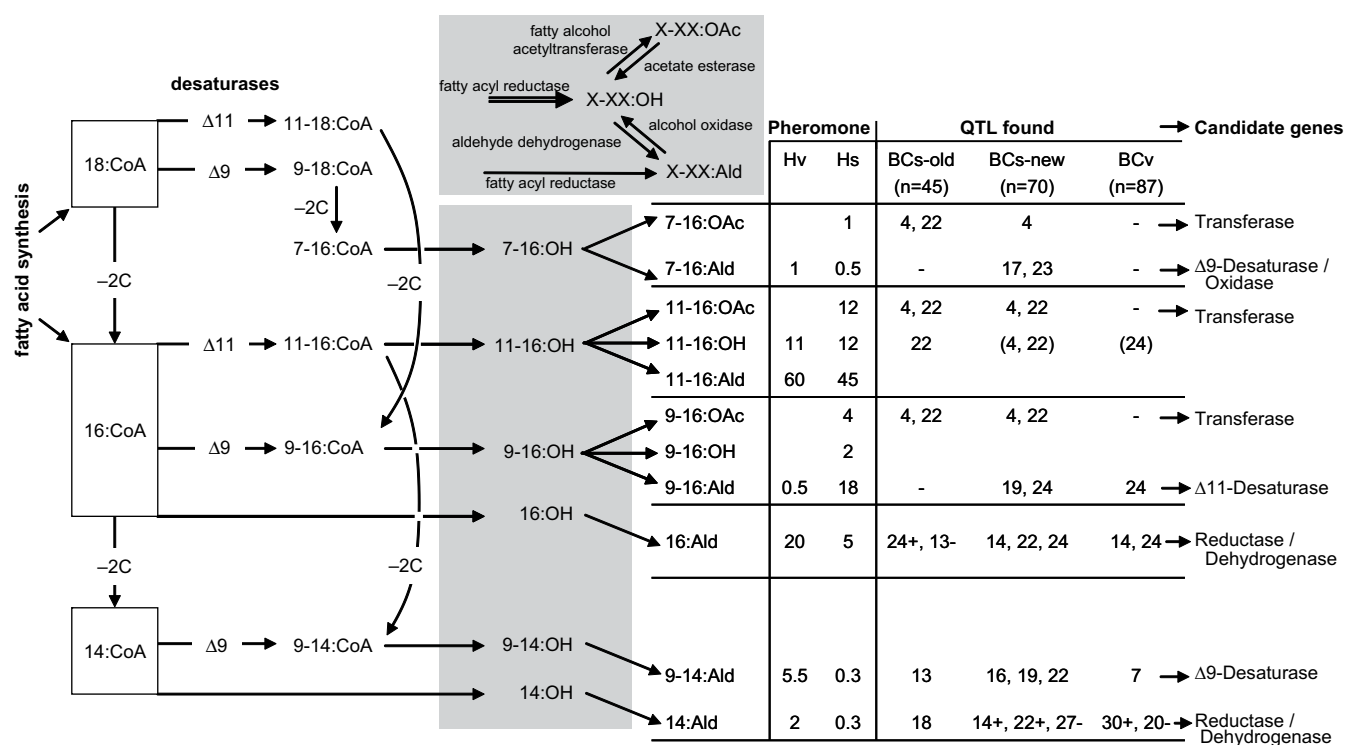


Fig. 1. Proposed pathways of the biosynthesis of the pheromone components in Hv and Hs (partly based on Jurenka, 2003; Choi et al., 2005), followed by the QTL found in Sheck et al. (2006) (BCs old) and in this study. The interplay of desaturation and chain-shortening of 18-, 16-, and 14-carbon acyl-CoA derivatives produce mono-unsaturated acyl-CoA precursors that are then modified to form alcohols, aldehydes, and acetate esters. The number that follows Δ indicates the position of the double bond introduced by the desaturase into the acyl-CoA. -2C indicates chain-shortening by two carbons through β-oxidation. The order of desaturation and chain-shortening results in different compounds. The precursor alcohols are shown in the vertical grey box, and the generalized pathways leading to aldehydes and acetate esters are represented in the upper grey box. Arrows from the alcohols lead to the compounds present in the pheromone glands of Hv and Hs. The numbers in the Pheromone columns refer to relative percentage of these compounds in the pheromone gland of Hv and Hs females, collected as larvae in the field in NC in 2004–2006 (see Groot et al., in press). The numbers in the QTL columns refer to chromosome numbers; + QTL in the expected direction, - QTL in the opposite-to-expected direction. The QTL for Z11-16:OH are in parenthesis because they are likely due to the pivotal role of Z11-16:OH as precursor to the major pheromone component (Z11-16:Ald), as well as the statistical interdependence between relative amounts of the different pheromone components.

pheromone component of Hs. The greater genetic detail in this study allowed us to combine the QTL data with information on the biosynthetic pathways of pheromone production, making possible predictions of candidate gene(s) for each QTL.

2. Materials and methods

2.1. Moth strains and backcross procedures

Single-pair matings were set up, hybridizing Hv females (YDK strain) with Hs males. The parental strains of Hv and Hs were the same as those described by Sheck et al. (2006), and had been in the lab for ~160 and ~40 generations, respectively. The F₁ females were backcrossed to Hs males (to which we refer as the S-backcrosses) and to Hv males (V-backcrosses). As did Sheck et al. (2006), we used two families per backcross: a candidate-QTL generating backcross, followed by a second backcross to confirm the candidate QTL from the first backcross, following Belknap et al. (1996). However, in this study the candidate-QTL generating backcross family in each direction consisted of >100 females. Also, since certain QTL may be dominant or recessive, this time we conducted backcrosses in both directions. In each direction, females that generated the two largest backcross families were chosen for subsequent analyses. In the S-direction, the two largest families consisted of 117 females (referred to as BCs1) and 70 females (BCs2). In the V-direction, the two largest backcross families consisted of 112 females (BCv1) and 87 females (BCv2). All insects were reared on artificial diet as described by Sheck and Gould (1993, 1995) at 27 °C, 70% RH, and a light–dark cycle of 14 h L: 10 h D.

2.2. Pheromone analysis and phenotypic correlations

The pheromone glands of backcross females were extracted and analyzed, following Groot et al. (2004), with minor modifications. Females were injected during the photophase with 1 pmol Hez-PBAN (Peninsula Laboratories, San Carlos, California) in 1 µl saline, using a 10 µl syringe with a 31 gauge needle (Hamilton, Reno, Nevada) that was inserted ventrally between the 8th and the 9th abdominal segments. Experimental injection of commercially available PBAN induces pheromone production within 2–3 h, independent of the time of day or the physiological state (mating status and age) of the female (Raina et al., 1989, 1991; Rafaeli, 2002; Groot et al., 2005), which thus minimizes the contribution of these variables to variation in the amount and blend ratio of the sex pheromone. One to 2 h after injection, the pheromone glands were dissected and extracted in conical vials containing 50 µl hexane and 20 ng of 1-pentadecyl acetate (gift from P. Teal) as an internal standard. After 20–60 min the glands were removed, and extracts were stored at –20 °C until analysis. The hexane extract was reduced to 0.5–1.5 µl under a gentle stream of N₂ and transferred along with 2 µl of *n*-octane into a 50 µl glass insert within an autoinjection vial capped with a Teflon lined crimp cap. The entire volume of each extract was injected in pulsed splitless mode (inlet at 250 °C) into a 5 m deactivated guard column coupled to a high resolution polar capillary column (DB-WAXetr [extended temperature range]; 30 m × 0.25 mm × 0.5 µm). The HP6890 gas chromatograph (GC) was temperature-programmed from 80 °C (1 min hold) to 150 °C at 10 °C/min, then to 225 °C at 3 °C/min, and finally to 250 °C at 20 °C/min, during which all the pheromone components eluted and Z7-16:Ald and Z9-16:Ald were adequately separated. Helium was used as the carrier gas at an average velocity of 31 cm/s (1.2 ml/min) and the flame-ionization detector was held at 250 °C.

The amount of each pheromone component was calculated relative to the 20 ng of internal standard. The pheromone components we quantified were: 14:Ald, Z9-14:Ald, 16:Ald, Z7-16:Ald,

Z9-16:Ald, Z11-16:Ald, Z7-16:OAc, Z9-16:OAc, Z11-16:OAc, and Z11-16:OH. The relative amount of each 'minor' component (i.e., all components other than Z11-16:Ald, which is the most abundant 'major' component in both species) was calculated as a percentage of all of the minor components combined to reduce the unexplained variance, following Sheck et al. (2006). A Pearson's correlation matrix was generated to determine phenotypic associations among pheromone components in the backcross females.

2.3. DNA extractions and AFLP marker analysis

DNA from individual grandparents, parents, and offspring was extracted, purified, ligated, pre-amplified, and selectively amplified after which AFLP fragments were separated based on size with an LI-COR sequencer (Lincoln, Nebraska, USA). Our AFLP protocol is described in detail by Sheck et al. (2006), and summarized here. The QIAGEN DNeasy 96 Tissue Kit, mouse tail protocol (QIAGEN, Valencia, California, USA) was used with some modifications after DNA was extracted from half of an adult thorax, which was approximately 20 mg of tissue. After restriction, the ligation step started with 20 µl of restricted DNA to which was added 0.5 µl of EcoRI adaptor (5 pmol/µl) (EcoRI top strand adaptor 5'-CTCGTAGCTGCGTACC-3', EcoRI bottom strand adaptor 5'-AATTGGTACG-CAGTCTAC-3'), 0.5 µl of MseI adaptor (50 pmol/µl) (MseI top strand adaptor 5'-GACGATGAGTCCTGAG-3', MseI bottom strand adaptor 5'-TACTCAGGACTCAT-3'), 0.5 µl ATP (10 mM, Invitrogen, Carlsbad, California, USA), 1 µl 5× Restriction/Ligation buffer, 0.5 Unit of T4 Ligase (Invitrogen), and 2 µl dH₂O for a final volume of 25 µl. The restricted, ligated DNA was diluted 1:10, which was pre-amplified using the AFLP Pre-Amp Primer Mix (LI-COR). The pre-amplified DNA was diluted 1:40 after which the selective amplification step followed. The core sequence of the E primer was 5'-GACTGCC-TACCAATTC-3', and the core sequence of the M primer was 5'-GATGAGTCTGAGTAA-3'. We added 3 selective bases to the end of each primer. AFLP fragments were separated based on size with an LI-COR 4200 sequencer that, with a scanning laser, simultaneously detected infrared labeled DNA fragments of 700 nm and 800 nm. The samples were run on an 8% polyacrylamide gel and loaded into 96 wells (0.1–0.4 µl per well) with a Hamilton 8-channel syringe (Hamilton, Reno, Nevada, USA). A labeled standard (LI-COR STR marker, 50–700 bp) was loaded in the first two (1–2) and last two (99–100) wells of the gel. The original parents and F₁ cross were always loaded into wells 3–6 and 95–98. The gels were run for 3 h and 15 min and the images were saved as a tiff files. We scored the gels using a semiautomatic image analysis program designed specifically for AFLP analysis (Quantar 1.08, KeyGene Products, Wageningen, The Netherlands).

The markers of interest in BCs1 and BCs2 were those that were present in the Hv parent and the F₁ female, but absent in the original Hs parent and the recurrent Hs backcross parent. Thus, the presence of a marker meant that the individuals were heterozygous (VS; having one copy from Hv and one copy from Hs), while the absence of a marker meant that the individuals were homozygous Hs (SS). In contrast, the markers of interest in BCv1 and BCv2 were those that were present in the Hs parent and the F₁ female, but absent in the original Hv parent and the recurrent Hv backcross parent. Here, the presence of a marker also meant that the individuals were heterozygous (VS), but the absence of a marker in this cross-specified homozygous Hv (VV).

2.4. Genetic map construction

For the construction of the linkage maps, we scored markers using all 117 BCs1 females and all 112 BCv1 females. In BCs1, 33 primer pairs

generated 108 markers, whereas in BCv1, 20 primer pairs generated 103 markers. At least two markers from different primer pairs were used to identify each chromosome. After identifying the 30 autosomes in these two families using MapMaker 3.0 (http://www.broad.mit.edu/genome_software/other/mapmaker.html), a subset of the primer pairs was chosen that generated AFLP markers that identified the presence/absence of all chromosomes in the other two families.

2.5. QTL analysis

A two-step method of QTL analysis, outlined in Belknap et al. (1996), was conducted following Sheck et al. (2006). This method is designed to reduce the type-I error associated with running a large number of statistical tests. It first uses one family to screen for candidate QTL, and then tests the effects of only these candidate QTL in a second family. In this way, false positives from the first family are unlikely to be significant in the second family and can be eliminated as candidate QTL, while QTL that are confirmed in the second family are unlikely to be significant by chance, and should be considered as robust QTL. This method circumvents the need for a Bonferroni correction because all tests in the second segregating family are *a posteriori* tests based on hypotheses from the first family results. The two-step QTL method has also become more frequently used in behavior genetics and in crop breeding because, in contrast to analyses that use single or combined segregating populations to both find QTL and estimate their effects sizes, simulation studies and experimental studies have shown that this method does not inflate estimates of locus-specific effect sizes (Beavis, 1994, 1998; Schon et al., 2004; Bennett and Carosone-Link, 2006).

Since heliothines have 30 autosomes and there is no recombination in female Lepidoptera (Heckel, 1993), our analysis enabled us to localize a QTL to a specific chromosome that should, on average, include approximately 3% of the insect's DNA. This level of resolution is similar to or finer than in many QTL analyses where recombination is present (e.g., Ting et al., 2001; Gleason and Ritchie, 2004; Moehring et al., 2004). The genome size of Hv is approx 403 MB as estimated by flow cytometry (Taylor et al., 1993). The length of linkage map is unknown for Hv or Hs, but has been determined as 2051 cM for closely related *Helicoverpa armigera* (Heckel et al., 1998). For the S-backcross, the BCs1 family was used to screen for candidate QTL, after which the effects of these candidate QTL were tested in the BCs2 family. For the V-backcross,

the BCv1 family was used to screen for candidate QTL, after which the effects of these candidate QTL were tested in the BCv2 family.

To assess whether there was a phenotypic effect of each chromosome at the 0.05 level of significance, data on each minor pheromone component were analyzed using ANOVA (PROC GLM in SAS, Version 9.1, 2002–2003) after checking all data for normality. The model separately tested the effect of presence/absence of a copy of each chromosome (Hv-chromosomes in the S-backcrosses and Hs-chromosomes in the V-backcrosses) on the relative amount of each of the minor pheromone components (excluding Z11-16:Ald). The R^2 values from these ANOVAs provided an estimate of the amount of phenotypic variation in the relative amount of a single pheromone component in backcross females that could be explained by presence/absence of one copy of a particular Hv or Hs-chromosome. All tests in the replicate families (i.e., BCs2 and BCv2) were one-tailed, because results from the first analyses preclude tests for differences in both directions. When more than one chromosome affected the relative amount of a specific compound, a three-way ANOVA was conducted to test for epistatic interactions between QTL on the chromosomes involved (fixed factors were family, chromosome identity, and presence/absence of the Hv or Hs copy).

3. Results

3.1. Phenotypic correlations

Since pheromone components are related to each other through common biosynthetic pathways (Jurenka, 2004; Fig. 1), we constructed a matrix of Pearson's correlations for all components in each of the 4 crosses (Tables 2 and 3). Here we concentrate on the strongest positive and negative phenotypic correlations ($P < 0.0001$).

3.1.1. S-backcrosses

In both the BCs1 and BCs2 families some main patterns emerged that shed light on the biosynthetic pathway through which pheromone components are most likely produced (Table 2). First of all, the three acetates were positively correlated with each other, confirming the results of our previous analysis (Sheck et al., 2006). The correlations between Z7-16:OAc and the other two acetates were lower in the current analysis than in Sheck et al. (2006), probably because of random quantification errors associated with the relatively low amount of Z7-16:OAc extracted from pheromone glands.

Table 2

Pearson's correlation coefficients for the S-backcross (to Hs males) families BCs1 and BCs2 when the sum of the **minor** components (i.e., excluding Z11-16:Ald) is set to 100%. Significant interactions are shown in bold. (Sequence of components is now the same as in Fig. 1.)

Family BCs1 (n = 117)	Z7-16:OAc	Z7-16:Ald	Z11-16:OAc	Z11-16:OH	Z9-16:OAc	Z9-16:Ald	16:Ald	Z9-14:Ald
Z7-16:OAc	—							
Z7-16:Ald	0.32***	—						
Z11-16:OAc	0.35****	-0.28**	—					
Z11-16:OH	-0.26**	-0.14	-0.61****	—				
Z9-16:OAc	0.42****	-0.13	0.87****	-0.59****	—			
Z9-16:Ald	-0.15	0.37****	-0.34***	-0.27**	-0.11	—		
16:Ald	-0.25**	0.19*	-0.50****	-0.12	-0.57****	0.02	—	
Z9-14:Ald	-0.005	0.27**	-0.41****	0.34***	-0.41****	-0.05	0.16	—
14:Ald	-0.06	0.27**	-0.39****	0.16	-0.43****	-0.11	0.43****	0.70****
Family BCs2 (n = 70)	Z7-16:OAc	Z7-16:Ald	Z11-16:OAc	Z11-16:OH	Z9-16:OAc	Z9-16:Ald	16:Ald	Z9-14:Ald
Z7-16:OAc	—							
Z7-16:Ald	0.20	—						
Z11-16:OAc	0.77****	-0.12	—					
Z11-16:OH	-0.39****	-0.13	-0.59****	—				
Z9-16:OAc	0.71****	-0.07	0.87****	-0.62****	—			
Z9-16:Ald	-0.001	0.48****	-0.14	-0.41***	0.14	—		
16:Ald	-0.55****	-0.18	-0.44****	-0.19	-0.48****	-0.17	—	
Z9-14:Ald	0.12	0.27*	-0.20	0.17	-0.31**	-0.08	-0.02	—
14:Ald	-0.09	0.27*	-0.31**	0.002	-0.39***	-0.03	0.31**	0.76****

* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$.

Table 3

Pearson's correlation coefficients for the V-backcross (to Hv males) families BCv1 and BCv2 when the sum of the **minor** components (excluding Z11-16:Ald) is set to 100%. Significant interactions are shown in bold. (Sequence of components is now the same as in Fig. 1.)

Family BCv1 (n = 112)	Z7-16:Ald	Z11-16:OH	Z9-16:Ald	16:Ald	Z9-14:Ald
Z7-16:Ald	—				
Z11-16:OH	-0.08	—			
Z9-16:Ald	0.28**	0.15	—		
16:Ald	-0.13	-0.88****	-0.58****	—	
Z9-14:Ald	-0.02	0.27****	0.15	-0.42****	—
14:Ald	0.10	-0.21*	-0.11	0.10	0.57****
Family BCv2 (n = 87)	Z7-16:Ald	Z11-16:OH	Z9-16:Ald	16:Ald	Z9-14:Ald
Z7-16:Ald	—				
Z11-16:OH	-0.13	—			
Z9-16:Ald	-0.12	0.16	—		
16:Ald	0.06	-0.90****	-0.55****	—	
Z9-14:Ald	-0.08	0.47****	0.12	-0.56****	—
14:Ald	0.09	-0.23*	-0.05	0.10	0.39***

* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$.

Secondly, the relative amounts of the aldehydes Z7-16:Ald and Z9-16:Ald were highly positively correlated ($P < 0.0001$), suggesting that these components are coupled in a common biosynthetic pathway, likely involving $\Delta 9$ -desaturase: Z7-16:Ald would be the product of chain-shortening of 18:Acid followed by $\Delta 9$ -desaturation, whereas Z9-16:Ald would result from $\Delta 9$ -desaturase acting on 16:Acid (Fig. 1). The last strong positive correlation was between the relative amounts of 14:Ald and Z9-14:Ald, suggesting, as above, that they are regulated through a common pathway originating from 14:CoA (Fig. 1). Conversely, we found a strong negative correlation between the relative amounts of 16:Ald (more abundant in Hv) and the three acetates (more abundant in Hs), suggesting that when more acetates were produced (more Hs-like phenotype), less 16:Ald was produced, and vice versa. Strong negative correlations were also found between Z11-16:OH and the relative amounts of four of the eight other compounds; these correlations might reflect the central role that alcohols serve as precursors for the other pheromone compounds (see Fig. 1).

3.1.2. V-backcrosses

As in the S-backcrosses, the relative amount of 14:Ald was again strongly positively correlated with the amount of Z9-14:Ald (Table 3). In addition, the relative amount of 16:Ald was strongly negatively correlated with Z9-14:Ald, Z9-16:Ald and Z11-16:OH. The negative correlation between Z9-14:Ald and Z11-16:OH suggests that Z9-14:Ald and Z11-16:OH are formed via the same pathway that originates in Z11-16:CoA (Fig. 1).

3.2. Genetic mapping

After the (arbitrary) assignment of the linkage groups through MapMaker, we were able to determine homologies between some linkage groups in the current S-backcrosses and those in our previous backcrosses (Sheck et al., 2006). This was done through comparison of common markers that defined the different linkage groups. In this way we were able to identify Hv-chromosomes 4, 22, and 24 of our previous backcrosses. Chromosomes 13 and 18 of the previous families did not have any AFLP marker in common with chromosomes on which QTL were found in the current S-backcrosses. However, this does not imply that these linkage groups are different. Further analysis (e.g., bulk segregant analysis) is needed to test their similarity.

3.3. Quantitative trait loci

In the hypothesis-generating backcrosses (BCs1 and BCv1) we found 15 candidate QTL (chromosomes) in BCs1 and 11 chromosomes

in BCv1 that appeared to affect the relative amounts of one or more of the minor pheromone components. In the replicate (i.e., confirming) backcrosses (BCs2 and BCv2) the effect of 9 of the chromosomes found in BCs1 were confirmed in BCs2, while in BCv2 the effect of 5 of the 11 chromosomes was confirmed. Only the confirmed QTL are depicted in Fig. 2 and described below. Detailed results on all QTL found in the 4 backcross families can be found in Appendix Tables 1 and 2.

3.3.1. S-backcrosses

In general, two to three QTL were found for each of the minor pheromone components (Fig. 2). The variance explained by each of these QTL ranged from 6% to 37%. Some QTL were found to affect multiple pheromone components. For example, Hv-chromosome 14 was associated with higher proportions of 14:Ald and 16:Ald (i.e., S-backcross females that were heterozygous (VS) for chromosome 14 contained significantly higher relative amounts of 14:Ald and 16:Ald than females that were homozygous (SS)). Hv-chromosome 19 affected the production of two compounds, Z9-14:Ald and Z9-16:Ald, with a double bond at the C9-position. Hv-chromosome 24 was inversely associated with the production of 16:Ald and Z9-16:Ald: females that were heterozygous (VS) for chromosome 24 contained significantly more 16:Ald and significantly less Z9-16:Ald compared to females that were homozygous (SS); this pattern was also found in our previous backcrosses (Sheck et al., 2006). This inverse correlation corresponded to the phenotypic correlation we found between these compounds (Table 2). Hv-chromosome 4 was associated with a higher percent of all three acetates in the blend (Fig. 2), and Hv-chromosome 22 affected the production of all minor Hv components, except Z7-16:Ald and Z9-16:Ald. However, when we omitted the three acetates from the analysis in the S-backcrosses and instead included the major sex pheromone component Z11-16:Ald, the previous association of Z11-16:OH with chromosomes 4 and 22 disappeared (data not shown). Hence, the QTL for Z11-16:OH are most likely due to a lack of independence between the relative amounts of the acetates and the alcohol.

Most of the QTL affected the production of pheromone components in the expected direction. That is, females with a copy of Hv-chromosomes 14, 15, 19, and 22 (thus heterozygous VS) contained higher relative amounts of the predominantly Hv compounds 14:Ald and/or Z9-14:Ald than did homozygous SS females. This is as expected, because these components are represented in higher proportions in Hv females than in Hs females. Also, the presence of Hv-chromosomes 14, 22, and 24 was related to higher relative amounts of 16:Ald, while the presence of Hv-chromosomes 19 and 24 was related to relatively less Z9-16:Ald, as expected, since the pheromone glands of Hv females produce much less Z9-16:Ald than Hs females (Groot et al., 2005; Sheck et al., 2006; see Fig. 1).

Contrary to expectation, the presence of Hv-chromosome 27 was associated with a relatively lower amount of the minor compound 14:Ald; that is, females that were heterozygous (VS) for chromosome 27 contained less 14:Ald than females that were homozygous SS. Because 14:Ald is produced in larger amounts in Hv than in Hs, we expected one Hv copy to result in higher amounts of this compound than two Hs copies (i.e., homozygous SS). Such a reverse effect was also found by Sheck et al. (2006) for Hv-chromosome 18. It remains to be determined whether these two QTL are the same linkage group. Surprisingly, we found two QTL for the production of Z7-16:Ald, a minor component in both Hv and Hs that is produced in similar relative amounts in both species. This was unexpected because a QTL is generally found only when there are large phenotypic differences between the parental strains.

No epistatic interactions were found for any of the QTLs that were associated with the same pheromone components, except for the association of Hv-chromosome 4 and Hv-chromosome 22 with the relative amounts of the three acetate esters, similar to Sheck et al.

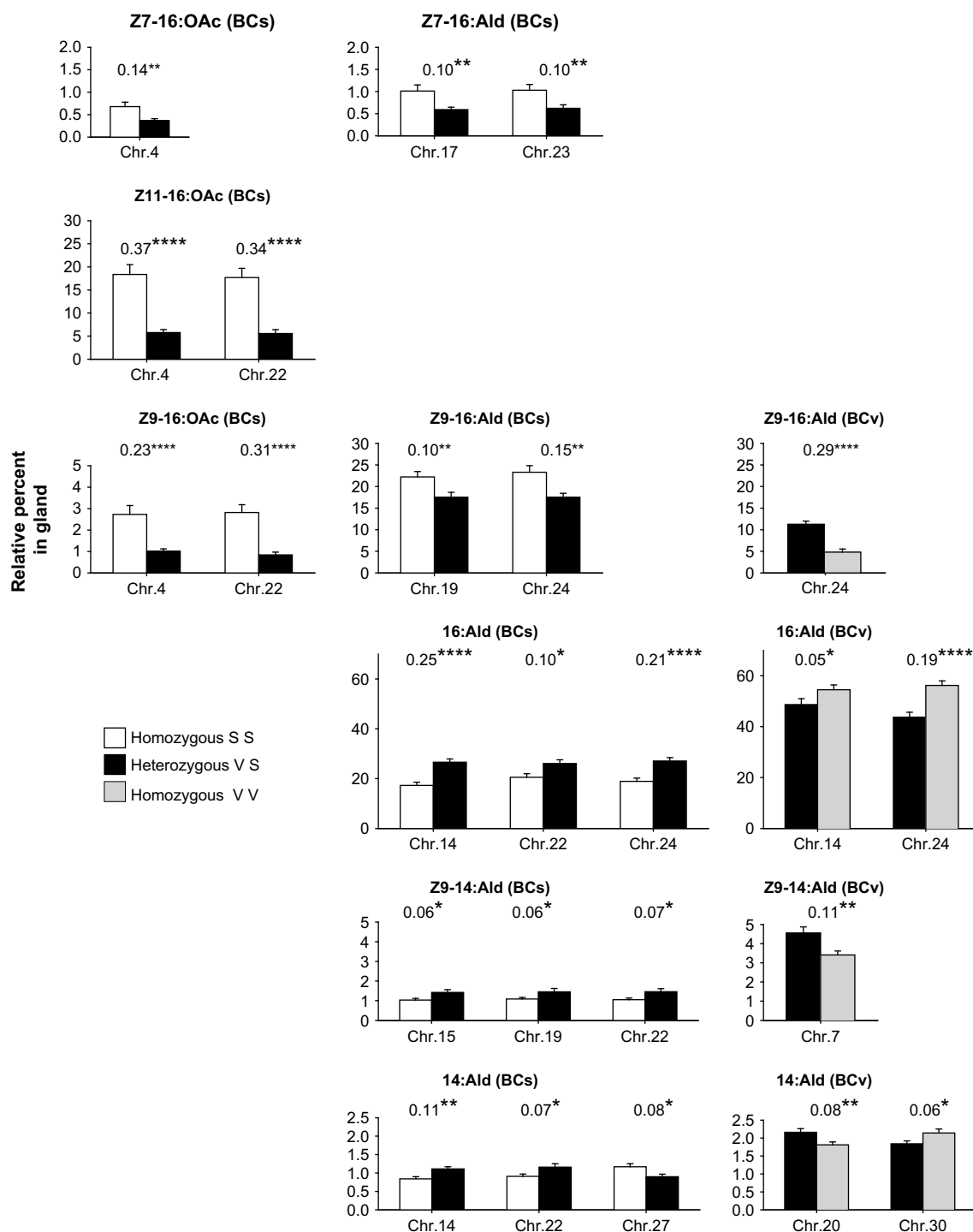


Fig. 2. QTL found in S-backcrosses (BCs) and in V-backcrosses (BCv), showing the effects of the presence/absence of each chromosome on the relative percentage of each pheromone component in the backcross females, when the sum of the minor components (excluding Z11-16:Ald) is set to 100%. Numbers above the bars are R^2 values of ANOVAs for the presence/absence of each chromosome in relation to the presence/absence of each of the minor pheromone components. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Note that chromosomes 7, 20 and 27 have an opposite-to-expected effect on the relative percentage of Z9-14:Ald in BCv, and 14:Ald in BCs and BCv, respectively. See text for further explanation. Variation is represented by +SEM.

(2006) (data not shown). The interaction for the acetates disappeared when the data were log transformed. As explained in Sheck et al. (2006), this indicates that the two chromosomes code for enzymes that act multiplicatively in reducing the relative amount of acetates.

3.3.2. V-backcrosses

QTL were found for all minor pheromone components, except for Z7-16:Ald, and explained 5–29% of the variance in BCv2 (Fig. 2).

Two QTL were found for 16:Ald: Hs-chromosome 14 explained 5% and Hs-chromosome 24 explained 19% of the variance in the relative amount of 16:Ald. Hs-chromosome 24 significantly affected the relative amount of Z9-16:Ald, explaining 29% of the variance (Fig. 2), as well as Z11-16:OH, explaining 7% of the variance (see Appendix Table 2). Phenotypically, Z9-16:Ald and Z11-16:OH were strongly negatively correlated with 16:Ald (Table 3). When we omitted 16:Ald from the analysis in the V-backcrosses and instead

included Z11-16:Ald, the previous association of Z11-16:OH with chromosome 24 disappeared. These results again confirmed our suspicion that the QTL found for Z11-16:OH were due to lack of independence of the relative amounts of the different pheromone components, i.e., an increase in one component necessarily implies a decrease in the other components. While this is true for all components, the central role of the alcohols in the biosynthetic pathway (see Fig. 1) makes them more prone to a dependent relationship with the other components.

Chromosome 24 showed the same effect in the V-crosses as in the S-crosses, namely an inverse correlation on the relative amounts of 16:Ald and Z9-16:Ald (this time, heterozygote females with one copy from Hv and one copy from Hs produced more 16:Ald and less Z9-16:Ald than homozygote VV females). Therefore, we assessed whether these linkage groups were the same in both backcrosses by developing a codominant marker for this chromosome based on sequencing one of the AFLP markers (M. Estock, unpubl. res). The presence of this marker in both linkage groups confirmed that these groups were the same.

The associations between presence or absence of Hs-chromosomes 14 and 24 and the relative amounts of 16:Ald and Z9-16:Ald were in the expected directions (Fig. 2), as Hs females produced more Z9-16:Ald and less 16:Ald than Hv females. Two QTL (Hs-chromosomes 7 and 20) that affected the relative amounts of 14:Ald and Z9-14:Ald had an opposite-to-expected effect; heterozygote SV females (i.e., females in which Hs-chromosomes 7 and 20 were present) produced significantly more of these components than homozygote VV females, while Hs females produced significantly less of these 14-carbon aldehydes than Hv females (see Fig. 2). A similar effect was found in the S-backcrosses with Hs-chromosome 27 as well as in C5–C6 families with chromosome 18 (Sheck et al., 2006). Most likely all these QTL are the same linkage groups, although we have not been able to find common markers for them. We are currently developing unambiguous markers to identify the chromosomes using SNP variation in housekeeping genes.

4. Discussion

To understand the evolutionary divergence of moth sexual communication systems, we have focused our research on determining the genetic basis of both female pheromone production and male response in two species with multi-component sex pheromone blends. Previously, we found five QTL affecting the production of one or more sex pheromone components (Sheck et al., 2006). The present study confirmed the specific QTL found in the previous study for 16:Ald and 14:Ald. Specifically, we found that chromosome 24 explained 21% of the variance in the relative amount of 16:Ald, which in our previous crosses explained 15% of the variance (Sheck et al., 2006). The second confirmed QTL was associated with the relative amount of 14:Ald. In the S-direction, backcross females with a copy of Hv-chromosome 27 (heterozygous VS) contained less 14:Ald than females without this Hv-chromosome (homozygous SS). Similarly, in the V-direction backcrosses, females with a copy of Hs-chromosome 20 (VS) had higher levels of 14:Ald than females without it (VV). This is surprising because in Hv this compound is produced in significantly higher relative amounts than in Hs. The most parsimonious explanation is that genetic drift has caused the divergence between Hv and Hs in this QTL (see Orr, 1998). It is also possible that the genes that regulate pheromone production have pleiotropic effects on other traits of the moths, and that selection on the other traits affected the divergence in the QTL. Another hypothesis is that a recessive cofactor increases production of this minor compound in heterozygote VS females when in a V-genomic background, but

decreases the production of 14:Ald in heterozygote VS females in an S-genomic background. Since Hs-chromosome 7 in the V-backcrosses also showed an opposite-to-expected effect on the production of the important secondary pheromone component Z9-14:Ald of Hv (i.e., VS females produced more than VV females, while the parental Hv females produced more than Hs females), such a cofactor may affect more than one pheromone component.

This study also confirms that Hv-chromosomes 4 and 22 affect the relative proportions of the acetates in pheromone glands, with chromosome 4 affecting all three acetates and chromosome 22 influencing Z9-16:OAc and Z11-16:OAc, the two most abundant acetates. In addition, we found new QTL for several minor components, specifically three more QTL for the production of 14:Ald (in the expected direction), three QTL for the production of 16:Ald, and two QTL for the production of Z7-16:Ald (see Fig. 2).

In our previous study we did not find a QTL for the critical pheromone component of Hs, Z9-16:Ald, at least not in the hypothesis-confirming backcross. Interestingly, we originally did find a large QTL for this component in the hypothesis-generating backcross, explaining 37% of the variance, but because it was not confirmed in the second backcross we had to dismiss it (Sheck et al., 2006). The fact that Sheck et al. (2006) could not confirm such a large QTL may have been due to the relatively small sample size. In the present study we found two QTL in both S-backcrosses and one QTL in the V-backcross for this component, which demonstrates the importance of biological replication. In the S-backcrosses these QTL were not very large: Hv-chromosome 19 and 24 explained 10% and 15% of the variance in the relative amount of Z9-16:Ald in BCs. However, in the V-backcrosses Hs-chromosome 24 explained 29% of the variance in Z9-16:Ald, which was the largest QTL found in these backcrosses. Apparently, in a V-genomic background this QTL has a much more pronounced effect than in an S-genomic background, which suggests some degree of dominance of this QTL in the V-backcrosses (or recessiveness in the S-backcrosses).

In summary, our QTL analysis was rigorous enough to confirm several QTL for most of the pheromone components. A larger sample size facilitated finding additional QTL, most importantly for Z9-16:Ald, the critical sex pheromone component of Hs. With these results it is possible to generate a list of candidate genes that may underlie these QTL by linking the established QTL to the biosynthetic pathway of the different sex pheromone components of Hv and Hs (see Fig. 1), with the cautionary note that the loci underlying these QTL may prove to be other than the genes encoding the enzymes themselves.

The overall phenotypic correlations among the acetates (Table 2) suggest that the accumulation of the three acetate esters in the S-backcrosses results from their common pathway involving reduction of the fatty acyl-CoA to the corresponding alcohol and conversion of the alcohol to acetate ester through acetyl transferase (Fig. 1). If an increase in the acetates is due to up-regulation and/or increase in specific activity of acetyl transferases, this enzyme is the candidate gene for the QTL represented by chromosome 4 and 22.

Chromosome 24 was a QTL for altering the relative amounts of Z9-16:Ald and 16:Ald in opposite directions. The most parsimonious explanation of this inverse correlation is that both are formed through 16:acid, and thus that Z9-16:Ald is formed via a $\Delta 9$ -desaturase (see Fig. 1). However, this contrasts with the findings of Choi et al. (2005) who reported greater incorporation of deuterium-labeled 18:acid than 16:acid into the Z9-pheromone components of Hs. Also, preliminary results with mapping the desaturases onto our AFLP map indicate that a $\Delta 11$ -desaturase and no $\Delta 9$ -desaturase maps to chromosome 24 (M. Ward, M. Estock, F. Gould, unpubl. res.). Hence, the strong inverse correlation of these components is more likely to be due to whether A) the 18:acid is converted by $\Delta 11$ -desaturase to Z11-18:acid (which is then further reduced to Z9-

16:acid), or B) the 18:acid is directly reduced to 16:acid, the precursor for 16:Ald (see Fig. 1). The gene underlying this QTL could thus encode a $\Delta 11$ -desaturase, the activation of which would lead to pathway A (Hs-like phenotype) while its inactivation would lead to pathway B (Hv-like phenotype).

The fact that chromosome 24 was also a QTL for altering the relative amounts of 16:Ald suggests that, in addition, a fatty acyl reductase and/or an aldehyde dehydrogenase may be located on this chromosome to convert the alcohol precursor 16:OH to the corresponding aldehyde. These candidate genes may also underlie the autosomal QTL for the production of 14:Ald and 16:Ald, as they convert 14:CoA and 16:CoA to the saturated compounds.

The strong negative phenotypic correlation between the relative amounts of 16:Ald (more abundant in Hv) and the two acetates, Z9-16:OAc and Z11-16:OAc (more abundant in Hs), suggests that when more acetates were produced (more Hs-like phenotype), less 16:Ald was produced, and vice versa. All three components can be produced via 16:CoA, which is either reduced to 16:Ald (an Hv trait) or desaturated (via $\Delta 9$ - and $\Delta 11$ -desaturases), then reduced and esterified to the acetate esters (which occur only in Hs). Therefore, competition for the 16:CoA precursor may explain the negative phenotypic correlation. The positive phenotypic correlation between the two saturated aldehydes (14:Ald and 16:Ald) supports the idea that when $\Delta 9$ -desaturases give rise to an Hs-like phenotype, not only less 16:Ald is produced, but also less 16:acid is chain shortened, producing less 14:Ald. In this way, regulation of 16:Ald and 14:Ald can be coupled. The fact that the two 14-carbon aldehydes are more abundant in Hv also supports common genetic regulation of their biosynthesis.

Chromosomes 17 and 23 were unique QTL in that they were only associated with the relative amount of Z7-16:Ald (in the S-backcrosses; see Fig. 2). Z7-16:Ald can only be formed via $\Delta 9$ -desaturation of the 18:Acid, followed by reduction of Z9-18:Acid to Z7-16:Acid (Fig. 1). Therefore, the candidate genes underlying these QTL are predicted to be a $\Delta 9$ -desaturase that has a substrate preference of 18:Acid over 16:Acid (such as NPVE, Knipple et al., 2002), and an oxidase that chain-shortens Z9-18:Acid to Z7-16:Acid.

Since QTL chromosome 19 was associated with the relative amounts of Z9-16:Ald as well as Z9-14:Ald, it seems likely that

another $\Delta 9$ -desaturase underlies this QTL. The strong positive phenotypic correlation between 14:Ald and Z9-14:Ald suggests that these components are regulated through a common pathway originating from 14:CoA and involving a $\Delta 9$ -desaturase as well, rather than Z9-14:Ald arising by $\Delta 11$ -desaturation of 18:CoA followed by chain-shortening.

The production of pheromone components via $\Delta 9$ -desaturases seems to be the ancestral state, as these desaturases are ubiquitous in many moth species (Knipple et al., 2002). Several $\Delta 9$ -desaturases have been characterized in Lepidoptera (Liu et al., 1999; Rosenfield et al., 2001; Knipple et al., 2002; Jeong et al., 2003; Park et al., 2008), facilitating the process of determining which $\Delta 9$ -desaturase gene(s) might be located within these QTL.

In conclusion, our findings in two independent studies that a number of QTL affect the production of almost all pheromone components in Hv and Hs indicate that the biosynthesis of each of these components is regulated by multiple enzymes in the biosynthetic pathway, and/or that the components are produced via multiple biosynthetic pathways. Importantly, several of the same QTL were revealed in both studies with associations with the same phenotypic effects. To determine which gene(s) underlie these QTL we are now fine-scale mapping these QTL and examining differential mRNA and protein levels in pheromone glands from females with and without specific introgressed chromosomes (Ward et al., in prep). In the specific cases where we can deduce candidate genes underlying the QTL, we are assessing whether and which of these genes map to the specific linkage groups (M. Ward, M. Estock, F. Gould, unpubl. res.).

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Appendix

Table 1

R^2 values of ANOVAs for the presence/absence of an Hv-chromosome (backcross to Hs males) in relation to the relative percentage of each of the minor pheromone components, when the sum of the minor components (i.e., excluding Z11-16:Ald) is set to 100%.

BCs1	C01	C04	C05	C09	C14	C15	C17	C19	C22	C23	C24	C26	C27	C28	C29
Z7-16:OAc		0.12***							0.03					0.04*	
Z7-16:Ald			0.04*				0.09**			0.06**					
Z11-16:OAc		0.17****						0.48****				0.04*			
Z11-16:OH	0.05*	0.05*							0.22****						
Z9-16:OAc		0.14****			0.07**				0.38****						
Z9-16:Ald			0.04*		0.08**			0.07**	0.06**		0.11***				
16:Ald	0.11***	0.10***			0.11***				0.07**		0.06**				0.04*
Z9-14:Ald					0.06*	0.04*	0.04*	0.04*	0.13****			0.04*		0.09**	
14:Ald	0.05*			0.04*	0.45****		0.04*		0.07**				0.05*		
BCs2	C01	C04	C05	C09	C14	C15	C17	C19	C22	C23	C24	C26	C27	C28	C29
Z7-16:OAc		0.14**													
Z7-16:Ald							0.10**			0.10**					
Z11-16:OAc		0.37****							0.34****						
Z11-16:OH		0.19***							0.12**						
Z9-16:OAc		0.23****							0.31****						
Z9-16:Ald								0.10**			0.15**				
16:Ald					0.25****				0.10*		0.21****				
Z9-14:Ald						0.06*		0.06*	0.07*						
14:Ald					0.11**				0.07*					0.08*	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Table 2
R² values of ANOVAs for the presence/absence of an Hs-chromosome (backcross to Hv males) in relation to the relative percentage of each of the minor pheromone components, when the sum of the minor components (i.e., excluding Z11-16:Ald) is set to 100%.

BCv1	C03	C05	C07	C11	C13	C14	C20	C21	C24	C25	C30
Z7-16:Ald				0.04*					0.05*		
Z11-16:OH		0.04*	0.04*				0.08**				
Z9-16:Ald									0.48****	0.07**	
16:Ald	0.06**						0.30****				
Z9-14:Ald			0.04*					0.05*			
14:Ald							0.06*	0.07**			0.04*
BCv2	C03	C05	C07	C11	C13	C14	C20	C21	C24	C25	C30
Z7-16:Ald											
Z11-16:OH									0.07*		
Z9-16:Ald									0.29****		
16:Ald						0.05*			0.19****		
Z9-14:Ald			0.11**								
14:Ald							0.08**				0.06*

* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

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