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Genetics of sex pheromone blend differences between *Heliothis virescens* and *Heliothis subflexa*: a chromosome mapping approach

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Abstract

Males of the noctuid moths, *Heliothis virescens* and *H. subflexa* locate mates based on species-specific responses to female-emitted pheromones that are composed of distinct blends of volatile compounds. We conducted genetic crosses between these two species and used AFLP marker-based mapping of backcross families (*H. subflexa* direction) to determine which of the 30 autosomes in these moths contained quantitative trait loci (QTL) controlling the proportion of specific chemical components in the pheromone blends. Presence/absence of single *H. virescens* chromosomes accounted for 7–34% of the phenotypic variation among backcross females in seven pheromone components. For a set of three similar 16-carbon acetates, two *H. virescens* chromosomes interacted in determining their relative amounts within the pheromone gland and together accounted for 53% of the phenotypic variance. Our results are discussed relative to theories about population genetic processes and biochemical mechanisms involved in the evolution of new sexual communication systems.

Introduction

The male and female components of long distance sexual communication systems are typically controlled by independent sets of genes (Butlin & Ritchie, 1989). Although there was preliminary support for a hypothesis that genes coding for mating-associated signals pleiotropically controlled the reception and response by the opposite sex (Hoy *et al.*, 1977), rigorous evidence for such pleiotropic genetic control in long distance auditory and chemical sexual signal/response systems is lacking (Butlin & Ritchie, 1989). When genetic correlations have been found between long distance mate-signalling traits in one sex and response to those signals in the opposite sex, the correlations appear due to linkage disequilibrium and not pleiotropy (e.g. Gray & Cade, 1999).

This independent genetic control of signals and responses raises evolutionary questions about how single mutations in such sexual signals or responses could increase in frequency (Butlin & Trickett, 1997). In some sexual communication systems, natural selection or sexual selection can result in a fitness advantage to rare genotypes that produce an altered signal or signal response phenotype, as in the case of tropical guppies where predators have selected for males with more cryptic coloration (Endler, 1991). In cases not involving predator escape or phenotypically biased sexual selection (e.g. unusually large ornaments coupled with biased mate choice), it is not easy to envision selective forces that favour an increase in the frequency of a rare genotype with a novel signal or response phenotype in face of the decreased probability of mating.

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Female moths typically emit a species-specific blend of two or more volatile pheromone components that attract conspecific males, which are only attracted to that blend (Cardé & Minks, 1997). Given the nocturnal activity of most moths, it is typical for long distance detection of appropriate mates to rely solely on a focused, efficient male response to a species-specific pheromone blend. Based on currently available data, a rare female that produces a novel pheromone blend is not expected to attract as many males as a normal female (Cossé *et al.*, 1995; Zhu *et al.*, 1997). Similarly, a rare male that responds most efficiently to a nonexistent or uncommon pheromone blend is expected to have low fitness relative to typical males. Without invoking external selective forces such as predators that are attracted to a species' common pheromone blend, or related moths that interfere with mating success of the common genotypes, it is difficult to envision how selection causes an initial increase in the frequency of rare genes for new moth mating signals or responses. Only one uncommon group of predators has been found to produce moth pheromones for hunting (Yeargan, 1994; Haynes *et al.*, 2003). Egg parasitoids that respond to moth pheromones are not solely responsive to a specific blend and are therefore unlikely to select for females with changes in a single sex pheromone component (e.g. Noldus & Van Lenteren, 1985; Reddy *et al.*, 2002). Under specific ecological conditions it may be possible for two populations that diverge in post-mating compatibility while in allopatry, to diverge in premating communication systems due to a reinforcement-like evolutionary process after gene flow is re-established (e.g. Sadedin & Littlejohn, 2003). Beyond appeal to these special selective forces, we must consider that for organisms with strong population structure, genes for novel signals and responses could increase due to genetic drift acting by chance against the effects of selection (Wright, 1931,1932; Wade & Goodnight, 1998; Peck *et al.*, 2000), or by drift in a specific environment where the novel blend was neutral.

With currently available data it is impossible to determine if the evolution of the diverse array of pheromone communication signals and responses in thousands of extant moth species arose through selection, drift, or a combination of the two. Population genetic theory indicates that an understanding of (i) the number of genes involved in the initial divergence, (ii) the magnitude of effect of each gene on fitness-related phenotypes and (iii) allelic interactions affecting fitness-related phenotypes, would be helpful in assessing the processes involved in evolution of particular systems (Butlin & Trickett, 1997; Coyne & Orr, 1998; Wade & Goodnight, 1998; Dieckmann & Doebeli, 1999; Kondrashov & Kondrashov, 1999; Whitlock & Phillips, 2000).

There is a growing body of data on the genetics of differences in sexual communication among moth races and among closely related moth species that provides preliminary information relative to the processes of evolutionary divergence (e.g. Löfstedt, 1993; Phelan, 1997; Roelofs & Rooney, 2003). However, most of this work has focused on a few races/species and on differences in ratios of geometric isomers of single pheromone components, and male response to these altered ratios (Phelan, 1997; but see Zhu *et al.*, 1997). The genetic crosses in these studies have typically implicated single genetic loci as causing altered female blend ratios and male responses (e.g. Löfstedt, 1993; Cossé *et al.*, 1995; LaForest *et al.*, 1997). In nature, many closely related species of moths differ not only in ratios of isomers but also in the presence/absence and amount of multiple compounds (Phelan, 1997; Witzgall *et al.*, 2004). Unfortunately, there is little information on inheritance of such differences (Haynes & Hunt, 1990; Gemeno *et al.*, 2001). Intriguing data supporting a single change in a desaturase gene causing appearance of a novel pheromone component within the genus *Ostrinia* (corn borers) was published recently (Roelofs *et al.*, 2002), and more studies of this kind on differences in distinct pheromone compounds are needed.

Our project focused on gathering data on the number of genes involved in qualitative and quantitative divergences between a pair of species in production of a number of distinct pheromonal compounds. We were also interested in determining potential pleiotropic effects of these genes and epistatic interactions between these genes. Our experimental system consisted of two distinct moth species, *Heliothis virescens* Fabricius (Hv) and *H. sublflexa* Guenée (Hs), that each produce a rich blend of pheromone components and differ from each other in the presence and absence of five compounds in the pheromone gland, and in the relative amounts of other, shared compounds (e.g. Pope *et al.*, 1982; Heath *et al.*, 1991; Teal & Tumlinson, 1997). These species differ dramatically in host range (Sheck & Gould, 1996), do not appear to be sister species (Fang *et al.*, 1997) and are at least sometimes sympatric (Klun *et al.*, 1982). There is preliminary evidence of intraspecific geographic variation in pheromone blends of Hv (Pope *et al.*, 1982; Ramaswamy & Roush, 1986; Heath *et al.*, 1990). Variation found among strains of Hs in pheromone blends could be due to intraspecific genetic variation or differences in methodology. For example, two aldehyde compounds, tetradecanal (14:Ald) and (*Z*)-9-tetradecenal (*Z*9–14:Ald), are typically not found in Hs pheromone glands, although Klun *et al.* (1979,1982) and Groot *et al.* (2005) have found traces of these compounds in the glands of Hs.

Hv and Hs produce seven and eight compounds in their pheromone glands, respectively. Wind tunnel and field trapping experiments indicate that two compounds are essential for male Hv attraction [i.e. (*Z*)-11-hexadecenal (*Z*11–16:Ald) and *Z*9–14:Ald] and three compounds are essential for Hs attraction [i.e. *Z*11–16:Ald, (*Z*)-9-hexadecenal (*Z*9–16:Ald), (*Z*)-11-hexadecen-1-ol (*Z*11–16:OH)] (Vetter & Baker, 1983; Ramaswamy *et al.*, 1985;

Heath *et al.*, 1990; Vickers, 2002). Most of the other emitted compounds have been shown to have a significant but less critical role in attraction (Teal *et al.*, 1986; Teal & Tumlinson, 1997).

Although these species are not attracted to each other's pheromone blends in the field (Klun *et al.*, 1982), they will mate with each other when placed in small containers. The F_1 female offspring of crosses between Hv females and Hs males are fertile (Karpenko & Proshold, 1977). F_1 males are sterile, but backcrossing to Hs restores male fertility (Karpenko & Proshold, 1977). Reciprocal F_1 crosses between Hv and Hs have provided initial information indicating that genes controlling the pheromone blends are not sex linked (Teal & Oostendorp, 1995). Preliminary data from Teal & Tumlinson (1997) on the pheromone composition of groups of F_1 and backcross females indicate almost complete dominance of the Hv phenotype for some components.

Our specific objectives in the present study were to (i) conduct single-pair crosses of F_1 hybrid females to Hs males, (ii) determine relative amounts of pheromone components in each of the segregating backcross female offspring, (iii) genetically map the segregating backcross populations, (iv) determine which of the 30 *Heliothis* autosomes contained quantitative trait loci (QTL) that controlled relative amounts of one or more components and (v) quantify the effect of QTL (and interactions between QTL) on the relative amounts of the pheromone components. Accomplishing these objectives was a first step toward our long-term goal of understanding how novel genes for altered pheromone production and male response could have evolved in noctuid moths.

Material and methods

Insect strains and backcrossing procedures

A colony of Hs, the South Carolina strain, was established by collecting larvae from *Physalis angulata* fruits. Forty-one larvae were collected on August 26, 1997 along roadsides and field margins near Barnwell and Florence, South Carolina. Larvae were transferred to a corn/soy meal artificial diet (Burton, 1970). If a larva fed on the diet, it was reared to pupation on the diet. If a larva did not feed on the diet, it was fed *P. angulata* fruits until pupation. Three larvae died, 21 pupae were females and 17 pupae were males. Adults were placed in 1 L buckets with sugar water wicks, and they were held in the greenhouse for mating and oviposition. *Physalis angulata* methanol extract was applied to the oviposition substrate (cheesecloth) to increase egg-laying. The next generation was established from 1200 neonates that were reared completely on artificial diet.

A colony of Hv, the YDK strain, was established in 1988 from a field collection in Yadkin County, NC (Gould *et al.*, 1995). It has been maintained continuously in the lab since then with a population size of approximately 250 adults per generation. Our experiments were conducted when the Hs and Hv strains had been in the laboratory for ca. 30 and 150 generations, respectively. Field experiments in 2004 show that females from these colonies are specifically attractive to their conspecific males (Groot *et al.*, unpublished data).

Two backcross families were created for the pheromone extraction and genetic analysis. Both hybrid families originated from a single Hv female (C) that was mated to a single-Hs male. Twenty-two of the F_1 hybrid daughters from this cross were backcrossed to Hs males in single-pair mating. Two of the most fecund backcross families (which are related as cousins) were chosen for the pheromone gland extraction. These families were named C5 and C6 because they were from daughters five and six of the original C female.

Pheromone extraction and gas chromatography (GC)

Female pupae from families C5 and C6 were placed individually in 30 mL plastic cups and were held in a light-controlled room with reversed photocycle. Scotophase began at 4 a.m. and ended at 2 p.m. No water or nutrients were provided to the emerged females. Two days after eclosion and 5–8 h into scotophase, cups containing single moths were placed on ice to slow down the females for easy handling. Pheromone glands were extruded from the abdomen either by pressing on the abdomen or by pulling on the ovipositor valves with a pair of fine forceps. The gland was then cut off with microdissection scissors, hemolymph and associated fat were blotted on a Kimwipe, and the gland was placed in a conical vial in 50 μL of hexane containing 20 ng of 1–pentadecyl acetate (from Peter Teal, USDA, Gainesville, FL, USA) as an internal standard. The gland remained in hexane for 10–20 min after which it was removed and discarded. The extract was kept at −20 °C until analysis. Pheromone gland extraction from 49 C5 backcross females and 46 C6 backcross females was conducted over the course of 2 weeks in June and July 2000. As a control, glands from 11 Hs females and 20 Hv females were extracted in July and August. Moths were frozen at −80 °C shortly after excision of the glands.

The hexane extracts were reduced to 1–2 μL under a gentle stream of high-purity N_2. The entire volume of extract was then injected into a splitless inlet (220 °C) of a HP6890 GC with a high-resolution polar capillary column (HP-20M, 50 m × 0.32 mm, 0.3 μm film thickness) and a flame-ionization detector (FID) that was held at 230 °C. Helium was used as carrier gas at constant pressure and an average velocity of 34 cm s^{-1}. The purge valve was opened 1 min after injection. The oven temperature was held at 80 °C for 2 min, then programmed to 210 °C at 20 °C min^{-1} and held at 210 °C for 15 min. The FID output was captured and processed on a HP-ChemStation (Version A.08.01) and the amount of each pheromone component was determined relative to

the internal standard. The identities of pheromone gland constituents were confirmed by comparison to retention times of authentic standards on polar (HP-20M) and nonpolar (DB-5) columns and some extracts were subjected to GC-mass spectrometry (Groot *et al.*, 2005).

Because there is extremely high variance among female moths in total pheromone gland content, even within treatments, most researchers report the amount of each component as either (i) a percentage of the single most abundant compound (i.e. the major component) (e.g. Heath *et al.*, 1991; Teal & Tumlinson, 1997) or (ii) a percentage of the total amount of all of the pheromone components in a gland (e.g. Heath *et al.*, 1991). We initially analysed our data using these two approaches. However, we found that in both cases variance in the major component, *Z*11–16:Ald, added noise to the data, and resulted in negative correlations between this major compound and most of the minor compounds. We found that when we eliminated *Z*11–16:Ald from our analysis and expressed the relative amount of each minor component (i.e. all compounds other than the most abundant compound are called minor components) as a percent of all of the minor components combined, the unexplained variance typically decreased. The major component is found in both species in generally similar amounts. Therefore, we felt that eliminating it from our analysis was not problematic. Throughout the paper we report 'relative amount' of each minor component as a percentage of the sum of all minor components in a pheromone gland. In the results section we also report the basic phenotypic statistics from the analysis that uses the sum of all components. More detailed analyses using percent of total of all compounds are reported in the appendix so that the reader can compare results of the two approaches.

We generated a matrix of Pearson's correlations to look for phenotypic associations among pheromone component quantities in the backcross females. Single-classification ANOVAS were used to test for differences between the two parent species and the two-backcross family females in relative amount of specific pheromone compounds. Coefficients of variation (CV) based on arc-sin square root transformed data were calculated for each compound in each female type.

DNA extraction

We used the Qiagen Qiamp DNA Mini Kit, mousetail protocol with some modifications. DNA was extracted from half of an adult thorax, which was approximately 20 mg of tissue. The minced tissue was placed in a labelled 1.5 mL microcentrifuge tube, and was frozen with liquid nitrogen before grinding with a chilled pestle that fit within the tube. Tissue was incubated overnight at 55 °C with 180 μL lysis buffer and 20 μL proteinase K per sample, then centrifuged at 12 000 $\times$ *g* for 5 min to precipitate the chitin. RNase A (3 μL at 4 μg μL^{-1}) was added to the supernatant and incubated at 37 °C for 15 min. The supernatant was adsorbed onto a column, washed with ethanol, and eluted from the column with 70 °C elution buffer. The final volume was 200 μL per sample. DNA was stored at −20 °C. DNA quality and amount in ng μL^{-1} were determined for each sample by running the samples on a 1% agarose gel and comparing to lambda standards. Samples typically had between 2.4 and 5 μg DNA per 20 mg of tissue. Genome size for *Heliothis* has been estimated at 4×10^8 bp (Taylor *et al.*, 1993). Therefore at 9×10^{11} bp ng^{-1}, we had approximately 5–10 million genome equivalents per individual.

AFLP markers

Our AFLP protocol was adapted from Remington *et al.* (1999) and Vos *et al.* (1995), and used infrared dyes for visualization on a Li-Cor sequencer. For the restriction step, we started with ≤300 ng of genomic DNA. For a total reaction volume of 30.0 μL, we added 6 units (U) of *Eco*RI, 8 U of *Mse*1 and 6 μL of 5 $\times$ R/L buffer (50 mM Tris HAc pH 7.5, 50 mM MgAc, 250 mM KAc, 25 mM DTT, BSA was added just before use). If necessary, volume was adjusted with sterilized deionized H_2O. The restriction digest was incubated for 2 h at 37 °C, after which we stopped the reaction by incubating at 70 °C for 15 min.

The ligation step started with 20 μL of restricted DNA from the previous step. For a total reaction volume of 25 μL, we added 0.5 μL of *Eco*RI adapter (5 pmol μL^{-1}), 0.5 μL of *Mse*I adapter (50 pmol μL), 0.5 μL ATP (10 mM), 1 μL 5 $\times$ R/L buffer, 1/2 unit of T4 Ligase (the adapters had the following sequences: *Eco*RI adapter 5′-CTCGTAGACTGCGTACC, 5′-AATTGGTACGCAGTC-TAC; *Mse*I adapter 5′-GACGATGAGTCCTGAG, 5′-TACT-CAGGACTCAT, these strands were annealed and had sticky ends). This reaction incubated overnight at 37 °C or room temperature. We then added 225 μL of sterilized deionized H_2O to the 25 μL of restricted, ligated DNA (R/L DNA) for a 1 : 10 dilution.

For the pre-amplification step, we started with 5 μL of the diluted R/L DNA. For a total reaction volume of 28 μL, we added 20.0 μL of pre-amp mix I (Gibco/BRL), 2.5 μL of 10 $\times$ PCR buffer + Mg (Boerhringer Mannheim) and 2.5 U of Taq polymerase. We used the following PCR amplification profile: 28 cycles, 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C. The pre-amplified DNA was diluted 1 : 40 by transferring 24 μL of the reaction product to a deep-well plate and adding 936 μL sterilized deionized H_2O. For future selective amplifications, the diluted DNA was plated out in 96-well plates (3 μL per well) and frozen at −20 °C.

Selective amplification used 3 μL of pre-amplified R/L DNA. For a total reaction volume of 12 μL, we added 3 μL of M primer (6 ng μL^{-1}), 1.2 μL of 10 $\times$ PCR buffer, 0.48 μL of dNTP, 5 mM, 0.14 μL of Taq polymerase (5 U μL^{-1}), 0.5 μL of IRD labelled E primer (Li-Cor) and sterilized deionized H_2O to bring up the volume. The

Table 1 Number of informative AFLP fragments scored per primer combination.

EcoRI primer	MseI primer	No. scored in C5	No. scored in C6
AAC	CAA	13	13
	CAC	7	9
	CAG	14	12
	CAT	26	25
	CCC	13	11
	CCT	29	27
	CGC	15	15
	CGT	16	15
	CTA	17	22
	CTC	18	16
AAG	CAG	17	13
	CAT	15	11
	CCA	16	15
	CCC	19	19
	CCG	11	11
	CCT	12	15
	CGC	26	30
	CGG	4	8
	CTA	15	16
	CTC	10	11
ACA	CAA	16	22
	CAC	8	6
	CCA	18	16
	CGA	19	16
	CTC	17	17
AGA	CCA	22	18
AGC	CAC	9	8
	CAG	10	10
	CCA	16	15
	CCT	15	15
	CGA	9	10
	CGG	8	8
	CTA	16	14
	CTT	18	22
AGG	CGA	18	14
Total markers		532	525

IRD primers are light sensitive and the reaction was kept covered with foil once they were added. The core sequence of the E primer was 5′-GACTGCGTACCAATTC and the core sequence of the M primer was 5′-GATGAGTCCTGAGTAA. We added three selective bases to the end of each primer. The selective bases we worked with are shown in Table 1. The PCR amplification profile was as follows: cycles 1–13: 10 s at 94 °C, 30 s at 65 °C; −0.7 °C per cycle; 60 s at 72 °C; cycles 14–36: 10 s at 94 °C; 30 s at 56 °C; 60 s at 72 °C + 1 s per cycle. We used a hot bonnet thermocycler (MJ Research).

AFLP marker analysis

AFLP fragments were separated based on size with a Li-Cor 4200 sequencer that, with a scanning laser, simultaneously detects infrared labelled DNA fragments of 700 and 800 nm. The samples were prepared for the polyacrylamide gels by adding 6 μL of formamide loading dye [95% formamide, 20 mM EDTA, bromophenol blue (USB)] per 12 μL reaction. Samples were denatured at 90 °C for 3 min and immediately placed on ice.

An 8% polyacrylamide gel was prerun for approximately 10–30 min set at 1500 V, 48 °C, background average : noise ratio approximately 2.5 : 0.5. The 96 samples (45 from C5 and 45 from C6 plus the grandparents and parents) were loaded into the wells (0.7–1.0 μL per well) with a Hamilton syringe. A labelled standard (Li-Cor STR marker, 50–700 bp) was loaded at each end. We loaded the 800-labelled samples first, ran the gel for approximately 5 min and then the 700-labelled samples were loaded in the same manner with the appropriate standard. The gels were run for about 3.5 h and the images were recorded in a computer file. We scored the gels using a semiautomatic image analysis program designed specifically for AFLP analysis (Quantar 1.08, KeyGene Products).

Mapping

The markers we considered of interest were those that were present as bands in gels from the Hv parent and the F_1 female, but were not present in the original Hs parent or recurrent Hs backcross parent, and were segregating in the backcross offspring in a 1 : 1 ratio as determined by a χ^2 analysis. Relaxing the segregation ratio requirement and adding males to the analysis (not shown) enabled us to identify the sex chromosome.

Because there is no crossing over in Lepidoptera females (Heckel, 1993), the chromosomes from the Hv female, C, used in the original F_1 cross should be inherited intact in both the C5 and C6 backcross offspring. In theory, all markers on a single chromosome will have a recombination fraction of zero, and therefore calculating map distances based on crossover frequency (centimorgans) would be inappropriate. Indeed, under ideal circumstances only a single marker would be needed for each of the 30 autosomes. In practice we find less than perfect correlations among markers for the same chromosome. This is most likely due to scoring error because map distance calculated for pairs of markers on the same linkage group are typically small, and the recombination values among markers were not correlated in two backcross families (R7 and R9) with the same grandmother (Sheck *et al.*, unpublished data). Because of the lack of perfect marker scoring, at least two markers from different primer pairs were used per chromosome. We determined linkage groups (i.e. chromosomes) with the mapping program, Mapmaker 3.0. Because we used a female Hv in the F_1 cross, and F_1 females in the backcrosses, all of the backcross females had a W sex chromosome from Hv and a Z sex chromosome from Hs. Therefore, any effects of sex chromosomes on the relative amounts of pheromone components could not be mapped. Such an analysis did

not appear to be needed based on the F_1 results of Teal & Oostendorp (1995) and preliminary backcross results of Teal & Tumlinson (1997) that did not find sex-linked inheritance.

Identification of chromosomes with alleles influencing pheromone blends

We followed the two-step method of QTL analysis outlined in Belknap *et al.* (1996). This method, which is designed to reduce the type-I error associated with running a large number of statistical tests, involves using one family to screen for candidate QTL and then testing for the effects of only those candidate QTL in a second family. In this way, any false positives from the first family are unlikely to be significant in the second family and can be eliminated as candidate QTL. Those QTL that are confirmed in the second family, however, are unlikely to be significant by chance, and should be considered to be robust QTL.

Our system with 30 autosomes and no recombination 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 that in many QTL analyses where recombination is present (e.g. Hawthorne & Via, 2001). Because the C5 and C6 backcross families were both derived from the same Hv grandmother, female C, the Hv chromosomes in both sets of backcross individuals were expected to share a high proportion of AFLP marker bands. This fact facilitated establishment of homology between the chromosomes that were separately mapped in the two-backcross families.

For the first step in our analysis, we screened the C5 family in order to tentatively identify chromosomes (linkage groups) containing QTL that controlled pheromone blends. To assess whether there was a phenotypic effect of each chromosome at the 0.05 level of significance, data on each minor component were analysed using ANOVA (PROC GLM in SAS, Version 8.01, 1999–2000). The model separately tested the effect of presence/absence of a copy of each Hv chromosome on the relative amount of each of the minor pheromone components in the C5 family females. 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 chromosome. Additionally, we tested for effects of each chromosome on the overall blend of minor components by using a MANOVA model (SAS, Version 8.01, 1999–2000). Those Hv chromosomes that had significant effects on the relative amount of one or more pheromone components, or on the overall blend, were hypothesized to contain pheromone-controlling QTL.

In the second and more stringent step, only chromosomes hypothesized to contain specific pheromone-controlling QTL, based on data from the C5 backcross family, were tested to determine if they also affected the relative amount of that specific pheromone component (or the overall blend) in the C6 backcross females. All hypothesis–testing regarding single components in the C6 family was one-tailed because results from the C5 analysis preclude tests for differences in two directions. For example, a candidate Hv allele that significantly decreased the relative amount of a component in the C5 family was only a candidate for causing a decrease in the C6 family. In this case, a statistically significant increase in C6 would have been considered an artifact. When more than one chromosome affected the relative amount of a specific compound, we used a three-way ANOVA to test for epistatic interactions between QTL on the chromosomes involved (fixed factors were family, chromosome identity and presence/absence of the Hv copy). We also used multiple linear regressions to determine the maximum proportion of phenotypic variation that could be explained by chromosomal differences among individuals.

Results

Pheromone composition

GC discretely separated a total of 8 of the 10 pheromone components from the two species and hybrids: tetradecanal (14:Ald), (*Z*)-9-tetradecenal (*Z*9–14:Ald), hexadecanal (16:Ald), (*Z*)-11-hexadecenal (*Z*11–16:Ald), (*Z*)-7-hexadecenyl acetate (*Z*7–16:OAc), (*Z*)-9-hexadecenyl acetate (*Z*9–16:OAc), (*Z*)-11-hexadecenyl acetate (*Z*11–16:OAc), and (*Z*)-11-hexadecen-1-ol (*Z*11–16:OH). Two other components, (*Z*)-7-hexadecenal (*Z*7–16:Ald) and (*Z*)-9-hexadecenal (*Z*9–16:Ald), were not consistently baseline-separated by our GC column and were combined for analysis. *Z*7–16:Ald is produced at low amounts in both moth species, so most of the difference in the combined value between the species and among backcross individuals is due to variation in *Z*9–16:Ald, which is much more abundant in Hs than in Hv.

The means, SEs and coefficients of variation for pheromone components of the parental species and the two-backcross families are presented in Tables 2 and 3. Table 2 gives values for each component as a percentage of the total amount of all of the pheromone components in a female gland. One compound, *Z*11–16:Ald (i.e. the major component) was always the most abundant pheromone component in both species and in the backcross females (Table 2). Table 3 gives values for each minor component as a percentage of the total amount of all the minor components (excluding *Z*11–16:Ald) in a female gland. Three acetates (*Z*7–16:OAc, *Z*9–16:OAc and *Z*11–16:OAc) were present in Hs and were absent in Hv. Other components were present in both species, but at different ratios. Three aldehydes (14:Ald, *Z*9–14:Ald and 16:Ald) were present at trace amounts in Hs females, but

Table 2 Means, SE's and CV's of each of the pheromone components, where the sum of all the pheromone components, including the major component Z11–16:Ald, is set to 100%.

	Hv ($n = 21$)			Hs ($n = 11$)			C5 ($n = 45$)			C6 ($n = 45$)		
Compound	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV
14:Ald	0.68	0.06	29	0.20	0.04	53	0.26	0.02	40	0.29	0.03	53
*Z*9–14:Ald	1.41	0.28	43	0.22	0.07	75	0.29	0.05	101	0.46	0.1	72
16:Ald	33.64	1.35	11	7.08	0.86	20	12.60	0.83	23	15.51	1.33	39
*Z*7/*Z*9–16:Ald	1.12	0.12	22	12.52	1.21	17	9.77	0.42	31	8.73	0.53	24
*Z*11–16:Ald	56.28	1.44	8	36.29	2.86	15	61.85	1.06	17	52.99	1.5	13
*Z*7–16:OAc	–	–	–	2.46	0.36	26	0.13	0.05	167	0.48	0.11	103
*Z*9–16:OAc	–	–	–	4.98	0.62	22	0.48	0.12	88	1.36	0.3	98
*Z*11–16:OAc	–	–	–	20.93	2.57	26	3.06	0.64	77	7.41	1.43	86
Sum acetates	–	–	–	28.37	3.39	25	3.67	0.79	77	9.25	1.82	88
*Z*11–16:OH	6.78	0.82	26	15.32	2.15	24	11.57	0.98	30	12.77	1.6	39

CV's were computed based on arcsin square root transformed data.

Table 3 Means, SE's and CV's of each of the minor pheromone components, where the sum of the minor components is set to 100%.

	Hv ($n = 21$)			Hs ($n = 11$)			C5 ($n = 45$)			C6 ($n = 45$)		
Compound	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV	Mean	SE	CV
14:Ald	1.57	0.14	16	0.33	0.07	54	0.69	0.06	35	0.67	0.08	47
*Z*9–14:Ald	3.26	0.63	36	0.36	0.11	73	0.74	0.09	37	0.97	0.19	50
16:Ald	77.17	1.94	8	11.60	1.71	25	32.95	1.83	23	34.07	2.81	41
*Z*7/*Z*9–16:Ald	2.78	0.26	19	20.88	2.93	25	26.97	1.63	23	19.79	1.49	30
*Z*7–16:OAc	–	–	–	3.74	0.44	20	0.29	0.10	165	0.91	0.18	100
*Z*9–16:OAc	–	–	–	7.64	0.75	18	1.18	0.24	87	2.61	0.52	95
*Z*11–16:OAc	–	–	–	31.92	3.26	23	7.55	1.43	74	14.10	2.41	83
Sum acetates	–	–	–	43.29	4.14	21	9.02	1.74	75	17.63	3.07	85
*Z*11–16:OH	15.23	1.59	16	23.54	2.56	19	29.6	1.84	24	26.88	2.70	38

CV's were computed based on arcsin square root transformed data.

in Hv their relative amounts were 5–10 times higher (in some other studies, e.g. Teal & Tumlinson, 1997, 14:Ald and *Z*9–14:Ald were reported as completely absent in Hs females). *Z*11–16:OH and the combined *Z*7– and *Z*9–16:Ald were higher in Hs than in Hv.

Unlike the parent species, the backcross females, typically, produced at least small amounts of all 10 compounds (with *Z*7–16:Ald and *Z*9–16:Ald combined as one component). As can be seen in Tables 2 and 3, there are some differences between the means for the C5 and C6 families. In some cases, our genetic analysis indicates that these differences are the result of stochastic variation in the number of offspring in each cross that inherited an Hv chromosome with a QTL for a specific pheromone component. The coefficient of variation (CV) in the relative amounts of some compounds was larger among the backcross females than among the individuals in either species (Tables 2 and 3). For each of the three acetates, the CV for the backcross females was more than 3–6 times as large as for the Hs females (Hv females do not produce acetates). This substantially larger CV in the genetically segregating backcross females is expected if one or a few loci control the amount of a pheromone component (Lande, 1981). For other pheromone components the CV for the backcross females were no larger than for the parents indicating polygenic inheritance.

Phenotypic correlations

Because pheromone components are related to each other through common biosynthetic pathways, we expected that the relative amounts of some components in the pheromone gland of a female would not be independent of each other, and that ratio of multiple components could be controlled by the same gene(s). Pearson's correlation analyses for the C5 (Table 4a) and the C6 (Table 4b) families show that the relative amounts of all three acetates have high-positive correlations ($P < 0.0001$), indicating that a similar pathway leads to accumulation of all of the acetates. Positive and negative correlations among other components are not as strong as among the acetates, but they may also be due to biosynthetic interrelationships. Because the relative amount of each component is recorded as a fraction of the total amount of all minor components, any physiologically independent increase in one component could

Table 4 Pearson's correlation coefficients for (a) Family C5 and (b) Family C6, when the sum of the minor components is set to 100%.

Family C5	14:Ald	*Z*9–14:Ald	16:Ald	*Z*7/*Z*9–16:Ald	*Z*7–16:OAc	*Z*9–16:OAc	*Z*11–16:OAc	Sum acetates
(a)								
14:Ald	1							
*Z*9–14:Ald	0.52***	1						
16:Ald	0.39**	0.03	1					
*Z*7/*Z*9–16:Ald	−0.09	−0.30*	−0.35*	1				
*Z*7–16:OAc	−0.13	−0.07	−0.25	−0.27	1			
*Z*9–16:OAc	−0.15	−0.14	−0.33*	−0.21	0.87****	1		
*Z*11–16:OAc	−0.19	−0.13	−0.24	−0.34*	0.83****	0.94****	1	
Sum acetates	−0.18	−0.13	−0.25	−0.33*	0.86****	0.96****	1.0****	1
*Z*11–16:OH	−0.19	0.29	−0.46**	−0.21	−0.32*	−0.38*	−0.39**	−0.39**
(b)								
14:Ald	1							
*Z*9–14:Ald	0.17	1						
16:Ald	0.46**	0.09	1					
*Z*7/*Z*9–16:Ald	0.2	−0.18	−0.18	1				
*Z*7–16:OAc	−0.32*	−0.16	−0.45**	−0.16	1			
*Z*9–16:Oac	−0.34*	−0.25	−0.45**	−0.23	0.86****	1		
*Z*11–16:OAc	−0.36*	−0.22	−0.44**	−0.31*	0.87****	0.95****	1	
Sum acetates	−0.36*	−0.22	−0.45**	−0.29	0.89****	0.97****	1.0****	1
*Z*11–16:OH	−0.22	0.37*	−0.44**	−0.02	−0.43**	−0.47**	−0.48***	−0.49***

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

cause a statistical decrease in another component. Therefore, some negative correlations in our data could simply be due to lack of independence between relative amounts of all components.

Genetic mapping

A total of 454 informative markers inherited from the original Hv female were found in both the C5 and C6 families. The C5 family had 102 unique informative markers, and the C6 family had 91 unique markers. The unique markers in the two families are expected because the initial female, C, was not highly inbred, so her daughters C5 and C6 were genetically distinct. We were able to identify 30 linkage groups (chromosomes) in C5 and 31 in C6 at a LOD level of 6.5. This included the sex chromosome. Linkage groups had no fewer than two markers (from different primer pairs) and as many as 26. The average number of markers per linkage group was 15.0 in C5 and 13.5 in C6. The two markers that identified the 31st chromosome in C6 were not present in C5. There were 83 markers in C5 and 106 markers in C6 that did not map to any linkage group. The sex chromosome linkage group could be specifically identified because all markers from this linkage group were present in the Hv parent, absent in the Hs parent and present in all of the backcross female offspring of C5 and C6.

From results in the hypothesis-generating step of the statistical analysis (i.e. single classification ANOVAS for the C5 family), 12 chromosomes appeared to affect the relative amount of one or more of the minor pheromone components (Table 5). Three chromosomes (1, 6 and 18) affected the amount of a single compound and nine

Table 5 r^2 Values of the ANOVAS for the presence/absence of an Hv chromosome (Chr) in relation to the relative percentage of each of the pheromone components, when the sum of the minor components is set to 100%.

Family C5	Chr01	Chr03	Chr04	Chr06	Chr08	Chr13	Chr16	Chr17	Chr18	Chr21	Chr22	Chr24
14:Ald					0.24**				0.46****	0.13*		
*Z*9–14:Ald						0.12*				0.16**		
16:Ald	0.13*					0.16**						0.35****
*Z*7/*Z*9–16:Ald				0.13*							0.11*	0.37****
*Z*7–16:OAc			0.17**		0.11*						0.30***	
*Z*9–16:OAc		0.10*	0.29***					0.11*			0.51****	
*Z*11–16:OAc		0.10*	0.23**				0.10*	0.11*			0.71****	
Sum acetates		0.10*	0.24***				0.09*	0.12*			0.67****	
*Z*11–16:OH						0.13*					0.17**	

d.f., 1, 39–40.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

chromosomes affected production of more than one compound. The number of chromosomes hypothesized to affect a particular compound ranged from 2 (i.e. *Z*11–16:OH) to 5 (i.e. *Z*11–16:OAc). MANOVA results indicated that chromosomes 4, 8, 13, 18, 22 and 24 had a significant effect on the overall blend (Chr4 $F_{8,33} = 3.06$, $P < 0.05$; Chr8 $F_{8,32} = 2.76$, $P < 0.05$; Chr13 $F_{8,32} = 2.89$, $P < 0.05$; Chr18 $F_{8,32} = 6.67$, $P < 0.0001$; Chr22 $F_{8,32} = 18.48$, $P < 0.0001$; Chr24 $F_{8,33} = 5.67$, $P < 0.0001$).

In the second step of the analysis, we tested whether the chromosomes hypothesized from the C5 analysis to contain QTL affecting specific pheromone components or the overall blend, also had significant impacts in C6 backcross females. Fourteen relationships between presence of a specific Hv chromosome and the relative amount of a minor pheromone component that were hypothesized from the C5 data were not confirmed in the C6 family. These were assumed to have been spuriously significant in C5 as an artifact of performing a large number of statistical tests. However, 11 hypothesized chromosomal effects from the C5 family were confirmed as impacting the C6 family (Table 6). There was a relationship between the *P* value for effects of a chromosome in C5 and whether it also had a statistically significant effect in C6. Of 12 hypothesized chromosomal effects based on a $0.05 > P > 0.01$ in the C5 family, only one was confirmed in the C6 analysis. Of six chromosomes with effects at $0.01 > P > 0.001$ in the C5, four were significant in the C6. All three chromosomes that were significant at the $0.001 > P > 0.0001$ in C5 were also significant in C6. Curiously, of six chromosomes that were significant at $P < 0.0001$ in C5, there was one that was not significant in the C6 family. MANOVA demonstrated that chromosomes 4, 13, 18, 22 and 24 had overall effects on the pheromone blend (Chr4 $F_{8,36} = 2.23$, $P < 0.05$; Chr13 $F_{8,32} = 2.51$, $P < 0.05$; Chr18 $F_{8,32} = 4.37$, $P < 0.01$; Chr22 $F_{8,35} = 3.76$, $P < 0.01$; Chr24 $F_{8,36} = 2.64$, $P < 0.05$).

The relative amounts of each of the three 16-carbon acetates in the C6 females were affected by chromosomes 4 and 22 (Table 6, Fig. 1). In addition to testing for chromosomes that affected the relative amounts of individual acetates, we tested for an effect on the relative amount of all three acetates combined. Again, chromosomes 4 and 22 had significant main effects. The presence/absence of one copy of chromosome 4 from Hv, and the presence/absence of one copy of chromosome 22 from Hv independently accounted for 10 and 23%, respectively, of the variance in percentage of total acetates in the pheromone gland. We performed a 3-factor ANOVA to test whether there was an interaction effect between chromosomes 4 and 22 in determining the relative amount of acetates in a female in each backcross family. There was a large interaction effect of the two chromosomes (Table 7a). The difference in

Table 6 Second test in family C6 to verify significant effects of the presence/absence of an Hv chromosome (Chr) on the relative percentage of each of the pheromone components, when the sum of the minor components is set to 100% (one-tailed ANOVAs).

	14:Ald		Z9–14:Ald			16:Ald		Z7/Z9–16:Ald			Z7–16:OAc			Z9–16:OAc			Z11–16:OAc			Z11–16:OH	
Chr	8	18	21	13	21	1	13	24	6	22	24	4	8	22	3	4	17	22	3	4	16	17	22	13	22
r^2	–	0.34	–	0.09	–	–	0.07	0.15	–	–	–	0.12	–	0.21	–	0.09	–	0.19	–	0.1	–	–	0.23	–	0.14
P	ns	<0.0001	ns	0.025	ns	ns	0.044	0.004	ns	ns	ns	0.011	ns	0.001	ns	0.028	ns	0.001	ns	0.02	ns	ns	0.0005	ns	0.005

d.f., 1, 36–43.

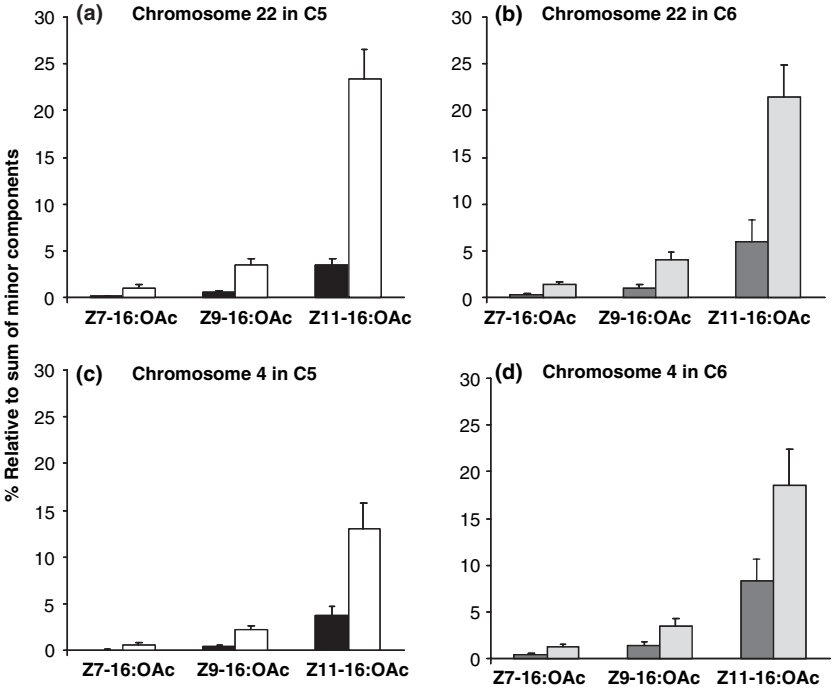

Fig. 1 Relative amounts of pheromone components in BC1 families C5 (1a,c) and C6 (1b,d) in relation to the presence (dark bars ± SE) or absence (light bars ± SE) of Hv-chromosome 22 (1a,b) and Hv-chromosome 4 (1c,d). Bars are mean percentages (±SE) where the total amount of the minor components is set to 100%.

Table 7 *P*-values of the three-way ANOVAS for (a) chromosome 4, chromosome 22 (b) chromosome 13, chromosome 24 and their interaction on the relative percentages of the acetates and of 16:Ald, as a percentage of the minor components, found in the glands of BC1 females (C5 and C6 together).

(a)

	*Z*7–16:OAc	*Z*9–16:OAc	*Z*11–16:OAc	Sum acetates
r^2	0.43	0.44	0.55	0.53
Chr4	0.0002	0.0003	<0.0001	<0.0001
Chr22	<0.0001	<0.0001	<0.0001	<0.0001
Chr4*Chr22	0.0019	0.0021	0.0013	0.0011

(b)

	16:Ald
r^2	0.30
Chr13	0.0066
Chr24	<0.0001
Chr13*Chr24	0.2715

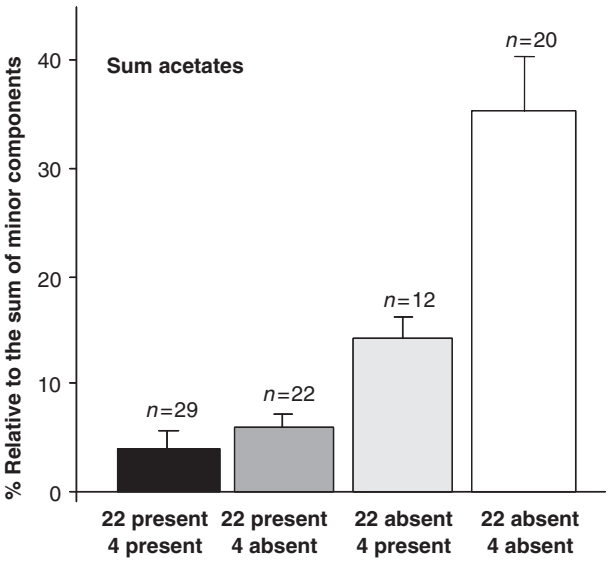

Fig. 2 Interaction effects on the production of acetates (*Z*7–16:OAc, *Z*9–16:OAc, and *Z*11–16:OAc combined) between chromosome 22 and chromosome 4 (C5 and C6 together). Bars are mean percentages (±SE) where the total amount of the minor components is set to 100%.

acetate content between individuals with zero and one Hv chromosome is greater than the difference between individuals with one and two Hv chromosomes (Fig. 2). When we log transformed the data before subjecting them to the 3-factor ANOVA we found no interaction effect. When both chromosome 4 and 22 are analysed together, they explain 53% of the phenotypic variance among C6 females in total acetates (a multiple linear regression for the relative amounts of all three acetates using $P < 0.01$ for adding new variables found that only chromosomes 4 and 22 contributed significantly, with a total of 56% of the variance explained. The difference between the two methods appears due to the multiple regressions using fewer observations).

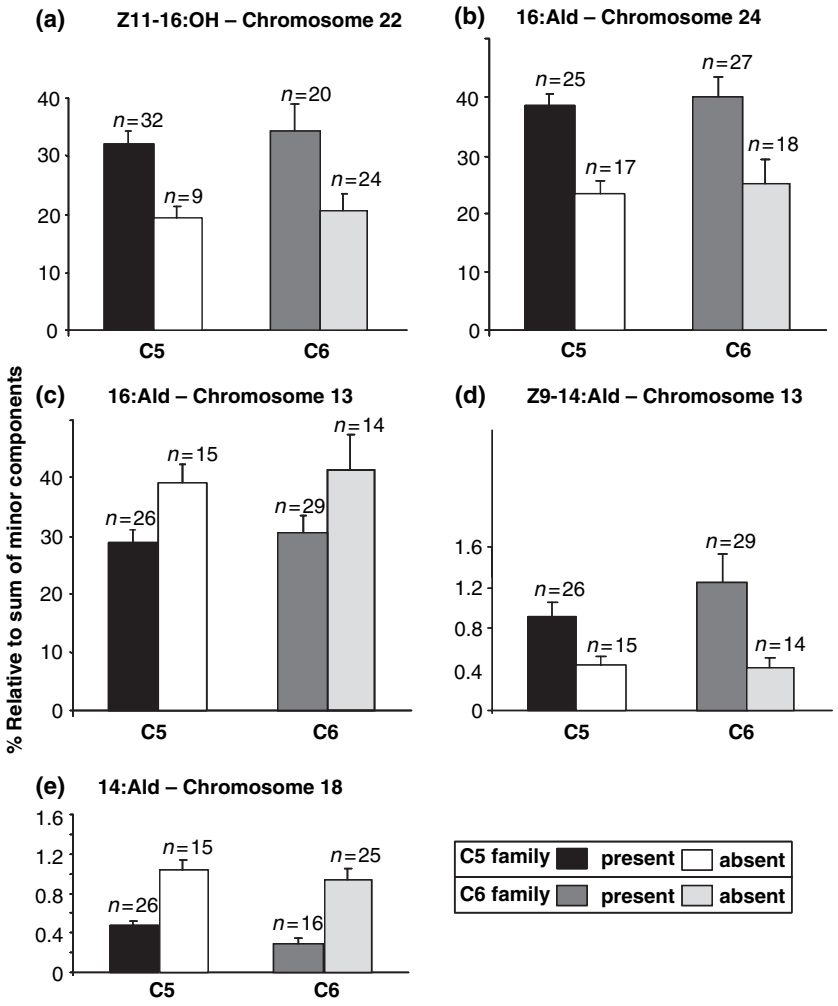

Fig. 3 Relative amount of pheromone components in BC1 families C5 and C6. Only graphs are shown for the pheromone components that significantly differed in relative amounts when an Hv chromosome was present or absent. Bars are mean percentages (±SE) where the total amount of the minor components is set to 100%.

Chromosome 22 from Hv that is associated with a decrease in acetates is associated with an increase in *Z*11–16:OH (Table 6, Fig. 3a). This result matches with the negative phenotypic correlation between *Z*11–16:OH and each of the three acetates (Table 4).

Hv females have higher relative amounts of 16:Ald than Hs females (Tables 2 and 3). The presence of one copy of chromosome 24 from Hv in backcross females resulted in higher amounts of 16:Ald than those found in females lacking this chromosome from Hv (Fig. 3b). The presence/absence of chromosome 24 from Hv accounted for 15% of the phenotypic variance in relative amount of 16:Ald (Table 6). Chromosome 13 from Hv had an opposite effect on 16:Ald from that caused by chromosome 24 and was unexpected (Fig. 3c). A 3-factor ANOVA indicated no interaction effect between the two chromosomes in determining the relative amount of 16:Ald with transformed or nontransformed data (Table 7b).

Presence of Hv chromosome 13 in C6 females resulted in a higher relative amount of *Z*9–14:Ald than found in females lacking this Hv chromosome and explains 9% of the phenotypic variance (Table 6, Fig. 3d). This is reasonable given the higher titer of this compound in the pheromone glands of Hv females compared to Hs females.

Chromosome 18 had a significant impact on the relative amount of 14:Ald in both families C5 and C6 (Table 6, Fig. 3e). In Family C6 it explained 34% of the phenotypic variance (Table 6). 14:Ald is present at higher ratios in Hv than in Hs. Surprisingly, the presence of one copy of chromosome 18 from Hv was associated with a lower relative amount of 14:Ald in the pheromone glands of C5 and C6 when it was expected to have the opposite effect.

Discussion

The long-term goal of our work is to understand how moth species have evolutionarily diverged from each other in sexual signal/response systems. To achieve this goal there is a need to understand the genetic basis of

variation in pheromone blends produced by female moths and variation in male response to divergent pheromone blends. Most studies related to this issue have focused on genetics of variation in ratios of two isomers of single pheromone compounds (e.g. Linn *et al.*, 1997), ratios of highly related compounds (Liu & Haynes, 1994; Foster *et al.*, 1997) and genetics of variation in male response to these altered ratios (Hansson *et al.*, 1989; Löfstedt *et al.*, 1989; Cossé *et al.*, 1995). These studies have been limited to determining if the major alleles controlling variation are located on autosomes or sex-determining chromosomes.

The current study differs from previous studies because it examines genetics of variation in multi-component blends that are typical of most moth species. Our study is in some ways preliminary, but it is the first work on long distance, sexual communication in any animal system (i) to map QTL affecting relative amounts of multiple sex pheromone components to specific autosomes, (ii) to assess the proportion of phenotypic variation in relative amounts of pheromone components that can be accounted for by the presence/absence of single chromosomes, (iii) to demonstrate that multiple pheromone components can be similarly or differentially affected by QTL on a specific chromosome and (iv) to demonstrate that QTL on multiple chromosomes can interact epistatically in affecting the relative amounts of pheromone components.

The conventional perspective regarding divergence in long distance mate finding systems assumes that females with a genetic alteration in the emitted blend ratio increase in frequency within a population and then the male response evolutionarily tracks this change (Phelan, 1997; Roelofs *et al.*, 2002). However, this perspective is problematic (Butlin & Trickett, 1997), because in the only well-studied systems (Glover *et al.*, 1991; Liu & Haynes, 1994; Linn *et al.*, 1997; Zhu *et al.*, 1997), females with altered ratios are much less attractive to conspecific males than normal females. It is, therefore, difficult to explain how the frequency of females with an altered blend initially increases in a population. Although most laboratory experiments indicate that males respond to a substantially broader range of ratios than produced by the females (e.g. Linn & Roelofs, 1995), in most cases the range of male response is clearly restricted and there is a narrow set of ratios that elicits peak response (Löfstedt, 1990; Linn & Roelofs, 1995). For example, in the redbanded leafroller, *Argyrotaenia velutinana*, the peak male response was to a blend of (*E*/*Z*)-11–14:OAc containing 7–9% *E*-isomer (Linn & Roelofs, 1995). Any female with a higher or lower percentage of the *E*-isomer would be expected to have a mating disadvantage, and the frequency of such females would not be expected to increase in the population unless some external ecological factors strongly selected for such a change (e.g. Raffa & Dahlsten, 1995) or stochastic events prevailed over selection (Wright, 1931). Experiments showing that some males in a population, at least sometimes, respond to a novel female blend (e.g. Roelofs *et al.*, 2002) are of interest. However, even if the response of these few males were genetically determined, their rare presence in the population would not be expected to significantly raise the fitness of females with the novel blend in comparison to typical females in the population (see Coyne & Orr, 2004, p. 226).

In contrast to the well-studied species and races in which only isomer ratios differ, many closely related moths, including Hv and Hs, differ in presence/absence of distinct compounds, and/or response to distinct compounds (Löfstedt, 1993; Witzgall *et al.*, 2004). It is important to ask whether the evolutionary addition of a new compound (or loss of an existing one) is as difficult to explain as the alteration in ratios of existing compounds. If a genetic alteration resulting in female production of a novel component causes a pleiotropic decrease in production of a critical existing component, that change may be selected against. Similarly, if a genetic change that results in male response to a nonexistent component causes decreased male sensitivity to critical components of the common pheromone blend, it will be selected against. More generally, if production of a new component had substantial physiological costs, an increase in frequency would not be expected.

Basic population genetic theory indicates that if production of a novel pheromone component was initially selectively neutral (i.e. had no effect on male attraction and no physiological cost), then a single mutation resulting in substantial production could become fixed in a population due to drift. This could then be followed by an increase in a mutation resulting in male response to the new component. In such a system, genetic analysis could be expected to reveal presence of major QTL. In contrast, if the production of the novel pheromone component by females were selected against until a novel genotype for male response to the new component became frequent, then a mutation resulting in substantial production of the new component would be chronically selected against. In such a situation a series of mutations for small increases in production and response might be more likely (Gavrilets, 2003), and these small changes could eventually add up to a major change in the pheromone system. The QTL in such a system would each have small effects and would be difficult to detect. Data on QTL for the pheromone components in our system at least generally fit with this hypothesis.

In our study we demonstrate that 7–34% of the phenotypic variation in each of seven pheromone components is accounted for by presence/absence of single copies of Hv chromosomes. For one component, *Z*9–16:Ald, no QTL were detected in the C6 family. The pheromone component for which a single chromosome explains the greatest percent of phenotypic variation (34%) is 14:Ald. This component is present in the glands of Hv (and at lower levels in Hs), and has been shown to

be present in the emitted pheromone blend of Hv (Heath *et al.*, 1991). However, this component has never been shown to attract or repel males of either species. At least based on our current understanding of mate attraction in these two species, variation in this component is not expected to be under strong selection pressure unless there is a physiological cost associated with its production. The fact that presence of an Hv chromosome in a backcross female causes a decrease in 14:Ald even though Hv itself has a higher titer of 14:Ald than Hs may be related to the lack of a selective function (Orr, 1998). Another compound 16:Ald has a small but significant impact on male attraction (Teal *et al.*, 1981; Vetter & Baker, 1983). This pheromone component is found in a higher relative amount in Hv than in Hs glands and has been shown to be in the pheromone plume of Hv (Heath *et al.*, 1991). For this component, one Hv chromosome causes an increase in relative amount while a second Hv chromosome causes a decrease (see Orr, 1998).

The acetates produced by female Hs are known to strongly deter Hv males (Vickers & Baker, 1997) and *Helicoverpa zea* (Fadamiro & Baker, 1997). These acetates cause a small increase in Hs male response to Hs females (Teal *et al.*, 1981; Vickers, 2002; Groot *et al.*, unpublished data). A major mutation in an Hs ancestor for production of acetates could have been neutral or positively selected for as a means to deter maladaptive encounters with other sympatric species. In light of this information, our finding of major QTL for acetate production on two chromosomes is not surprising.

In contrast to our results with the components discussed above that may not be strongly selected against when rare, the QTL for components that are essential for conspecific male response have smaller effects or are undetected. For *Z*9–14:Ald that is essential for mate finding in Hv (Vetter & Baker, 1983) the QTL found explains only 9% of the variance among C6 females. For *Z*9–16:Ald that is essential for mate finding in Hs (Vickers, 2002) we found no significant QTL in the C6 family. Not finding an association between presence/absence of any Hv chromosomes and this critical component suggests that this component is controlled by alleles on many chromosomes, with each having a very small effect that is hard to detect. We considered this possibility cautiously because of our small sample size and the fact that in the C5 cross Hv chromosome 24 had a significant effect on the *Z*9–16:Ald. We examined the C6 data for outliers that may have affected the results and found none. It was, therefore, important to ask how small a QTL we could have detected with our C6 data on *Z*9–16:Ald. Using the within class variance values in *Z*9–16:Ald of females with and without Hv chromosome 24, we determined that if chromosome 24 had explained more than 6% of the phenotypic variance we would have detected an effect at the $P < 0.05$ significance level. Our data, therefore, indicate that QTL for two essential pheromone components, *Z*9–16:Ald and *Z*9–14:Ald, appear to explain 9% or less of the phenotypic variance while for components with less critical effects QTL explain up to 34% of the phenotypic variance. It is tempting to speculate that sudden large increases in *Z*9–16:Ald and *Z*9–14:Ald were selected against and that the differences between the two species arose through an accumulation of smaller changes.

The component *Z*11–16:OH, requires separate consideration. This component is essential for attraction of Hs males (Vickers, 2002) and may repel sympatric *H. zea* males (Quero & Baker, 1999). Both Hs and Hv females contain this component in their pheromone glands but only Hs females emit this component in their pheromone plume (Heath *et al.*, 1991). It is thought that enzymes in the cuticle of Hv pheromone glands metabolize *Z*11–16:OH before it is emitted. Therefore, an evolutionarily meaningful QTL analysis for this component would require a future analysis of the volatiles in female pheromone plumes, which is feasible but labour intensive.

Although the evolutionary factors discussed above suggest why some pheromone components are strongly affected by single QTL while others are not, it is also possible that these differences in genetic control have biochemical explanations. While our understanding of pheromone biosynthetic pathways in Heliothines is better than in most organisms (e.g. Tillman *et al.*, 1999; Rafaeli, 2002; Jurenka, 2003) it is far from complete and it leaves uncertainty as to how many enzymatically-controlled biochemical reactions affect the amount of each pheromone component. The positive and negative phenotypic correlations between components in our study (Table 4a,b) shed some light on these biochemical interactions. Of most interest, we found that the relative amounts of all three acetates are positively correlated. This suggests that the same biochemical pathways affect all of them. The fact that there are three acetates in the Hs pheromone blend, instead of only one acetate, may be more related to the biochemical processes involved in production of acetates than to any specific sexual selection process. In the QTL analysis, both chromosome 4 and 22 of Hv caused a decrease in each of the three acetates. We also found epistasis between these chromosomes in their effects on the relative amounts of acetates, with the presence of Hv chromosome 22 causing a greater linear decrease in acetates when Hv chromosome 4 is absent than when it is present. The fact that this epistatic interaction disappeared when the data were log transformed indicates that the two chromosomes code for enzymes that act multiplicatively in reducing the relative amount of acetates. This would be expected if the QTL on the two chromosomes were duplicated genes or distinct genes that produced enzymes with similar activities.

Quantifying the effects of QTL on relative amounts of pheromone components is only a first step in under-

standing the evolution of moth sexual communication systems. Future studies must determine the impact of each QTL on the actual mating success of females. We have begun this approach by moving each single QTL, from the moth species in which the QTL product is found, into the genetic background of the moth species in which it is not found or is at reduced amounts (Groot *et al.*, 2004). It will also be important to determine the impacts on male mating success of single QTL that alter the male response to specific blends. Finally, it will be necessary to clone the specific genes involved in sexual communication so that we can determine how many base pair changes in each gene contribute to alterations in male and female traits.

The genetic tools available for accomplishing these goals in moth species are limited, but growing. It could be argued that it would be best to first understand the evolutionary genetics of sexual communication in model organisms such as *Drosophila* before embarking on the study of moths. However, moths have a major advantage over many other taxonomic groups when studying the evolution of sexual communication because moths are so heavily reliant on one specific modality for mate finding, and the organs involved in sexual communication, the pheromone glands and the antennae, are so well defined. Therefore, we believe that parallel study of moths and other organisms is likely to be the most efficient path to progress in this area.

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Appendix

This appendix contains phenotypic correlations and genetic analyses based on data including the minor pheromone components as well as the major pheromone component, *Z*11–16:Ald. The analyses presented in the article itself are based only on data from the minor components. An explanation for this approach is provided in 'materials and methods' section.

Table A1 Pearson's correlation coefficients for (a) Family C5 and (b) Family C6, when the sum of all components is set to 100%.

Family C5	14:Ald	*Z*9–14:Ald	16:Ald	*Z*7/*Z*9–16:Ald	*Z*11–16:Ald	*Z*7–16:OAc	*Z*9–16:OAc	*Z*11–16:OAc
(a)								
14:Ald	1							
*Z*9–14:Ald	−0.51***	1						
16:Ald	0.42**	0.16	1					
*Z*7/*Z*9–16:Ald	−0.32*	−0.38**	−0.51***	1				
*Z*11–16:Ald	−0.13	−0.44**	−0.41**	0.44***	1			
*Z*7–16:OAc	−0.06	−0.02	−0.12	−0.14	−0.37*	1		
*Z*9–16:OAc	−0.08	−0.08	−0.15	−0.15	−0.34*	0.92****	1	
*Z*11–16:OAc	−0.10	−0.08	−0.08	−0.28	−0.37*	0.89****	0.96****	1
*Z*11–16:OH	−0.05	0.50***	−0.13	−0.24	−0.60***	−0.16	−0.22	−0.21
(b)								
14:Ald	1							
*Z*9–14:Ald	0.10	1						
16:Ald	0.33*	−0.17	1					
*Z*7/*Z*9–16:Ald	0.03	−0.27	−0.35*	1				
*Z*11–16:Ald	0.37*	−0.22	0.01	0.34*	1			
*Z*7–16:OAc	−0.28	−0.09	−0.3*	−0.14	−0.53***	1		
*Z*9–16:OAc	−0.29*	−0.17	−0.34*	−0.10	−0.50***	0.90****	1	
*Z*11–16:OAc	−0.31*	−0.13	−0.29	−0.17	−0.61****	0.92****	0.96****	1
*Z*11–16:OH	−0.31*	0.52***	−0.37*	−0.17	−0.38*	−0.25	−0.31*	−0.27

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

Table A2 Family C5: r^2 values of the ANOVAS for effects of the presence/absence of an Hv chromosome (Chr) in relation to the relative percentage of each of the pheromone components, when the sum of all components is set to 100%.

Family C5	Chr01	Chr04	Chr06	Chr08	Chr12	Chr13	Chr16	Chr17	Chr18	Chr21	Chr22	Chr24
14:Ald				0.25***					0.43****	0.14*		
*Z*9–14:Ald										0.11*		
16:Ald	0.12*					0.23**						0.39****
*Z*7/*Z*9–16:Ald												0.45****
*Z*11–16:Ald					0.13*							
*Z*7–16:OAc		0.15*	0.10*								0.29***	
*Z*9–16:OAc		0.23**						0.10*			0.50****	
*Z*11–16:OAc		0.20**					0.09*	0.11*			0.66****	
Sum acetates		0.20**						0.10*			0.63****	
*Z*11–16:OH					0.11*							

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

Table A3 Second test of Family C6 to verify significant effects of the presence/absence of an Hv chromosome (Chr) on the relative percentage of each of the pheromone components, when the sum of all components is set to 100% (one-tailed ANOVAS).

	14:Ald			Z9–14:Ald			16:Ald			Z7/Z9–16:Ald	Z7–16:OAc			Z9–16:OAc			Z11–16:OAc				Z11–16:OH
Chr	8	18	21	21	1	13	24	24	4	6	22	4	17	22	4	16	17	22	12
r^2	–	0.34	–	–	–	0.08	0.12	–	0.12	–	0.12	0.1	–	0.17	0.1	–	–	0.19	–
P	ns	<0.0001	ns	ns	ns	0.025	0.01	ns	0.01	ns	0.003	0.02	ns	0.003	0.021	ns	ns	0.002	ns