*Journal of Chemical Ecology, Vol. 30, No. 12, December 2004 (© 2004)*

# INTROGRESSING PHEROMONE QTL BETWEEN SPECIES: TOWARDS AN EVOLUTIONARY UNDERSTANDING OF DIFFERENTIATION IN SEXUAL COMMUNICATION

ASTRID T. GROOT,[1,*] CATHERINE WARD,[1] JING WANG,[1] AMANDA POKRZYWA,[1] JENNIFER O'BRIEN,[1] JOY BENNETT,[1] JENNIFER KELLY,[1] RICHARD G. SANTANGELO,[1] COBY SCHAL,[1] and FRED GOULD[1]

[1]*Department of Entomology and W. M. Keck Center for Behavioral Biology North Carolina State University, Raleigh North Carolina 27695–7613, USA*

(Received August 6, 2004; accepted September 20, 2004)

**Abstract—**As a first step toward understanding how noctuid moths evolve species-specific pheromone communication systems, we hybridized and backcrossed two closely related moth species, *Heliothis virescens* (Hv) and *H. subflexa* (Hs), which differ qualitatively and quantitatively in their multicomponent sex pheromone blends. We used amplified fragment length polymorphism (AFLP) marker-based mapping of backcross families to determine which of the 30 autosomes in these moths contained quantitative trait loci (QTL) controlling the percentages of specific chemical components in the pheromone blends. In two previous backcrosses to Hs, we found a strong depressive effect of Hv-chromosome 22 on the percentage of three acetate components in the pheromone gland. These acetates are present in Hs and absent in Hv. Here, we describe how we introgressed Hv-chromosome 22 into the genomic background of Hs. Selection for Hv-chromosome 22 started from backcross 3 ($BC_3$) females. All females that had Hv-chromosome 22 and a low percentage of acetates (<3% of the total amount of pheromone components present) were backcrossed to Hs males. In $BC_5$ to $BC_8$, we determined whether Hv-chromosome 22 was present by a) running only the primer pairs that would yield the markers for that chromosome, and/or b) determining the relative percentages of acetates in the pheromone glands. Either or both genotype and phenotype were used as a criterion to continue to backcross these females to Hs males. In $BC_9$, we confirmed the isolation of Hv-chromosome 22 in the Hs genomic background, and backcrossed the males to Hs females to eliminate the Hv-sex chromosome as well as mitochondrial DNA. The pheromone composition was determined in $BC_3$, $BC_5$, and $BC_{11}$ females with and without

* To whom correspondence should be addressed. E-mail: astrid_groot@ncsu.edu

Hv-chromosome 22. All backcross females with Hv-chromosome 22 contained significantly less acetates than females without this chromosome. In addition, $BC_3$ females with Hv-chromosome 22 contained significantly more *Z*11-16:OH than $BC_3$ females without Hv-chromosome 22. However, in $BC_5$ and $BC_{11}$ females, the correlation between *Z*11-16:OH and Hv-chromosome 22 was lost, suggesting that there are separate QTL for the acetates and for *Z*11-16:OH, and that the relative amount of the alcohol component is only affected in epistasis with other (minor) QTL. Now that we have succeeded in isolating the chromosome that has a major effect on acetate production, we can test in behavioral experiments whether the presence of acetates may have been a driving force for a shift in pheromone composition. Such tests are necessary to move towards an evolutionary understanding of the differentiation in sexual communication in *Heliothis* spp. moths.

**Key Words—***Heliothis virescens*, *Heliothis subflexa,* multi-component sex pheromone blend, QTL, quantitative trait loci, backcross, AFLP, amplified fragment length polymorphism, phenotype, genotype.

## INTRODUCTION

The number of animal species for which we have even a rudimentary understanding of the genetic control of sexual communication is small, but the few detailed studies involve insect species (Löfstedt, 1990, 1993; Butlin, 1995; Linn and Roelofs, 1995; Phelan, 1997; Coyne and Orr, 1998; Roelofs and Rooney, 2003). Early genetic studies of sexual communication systems focused on determining if the same genes that controlled signal production also controlled signal perception in the opposite sex through pleiotropic effects (sometimes referred to as genetic coupling). Although an early empirical study found evidence supporting the possibility of genetic coupling of acoustic mate communication (Hoy et al., 1977), other studies of both acoustic and chemical sexual communication indicate that such coupling is rare (reviewed in Butlin and Ritchie, 1989). One study that found a genetic correlation between male and female signal and response traits in offspring from field-collected insects determined that these correlations broke down after randomized mating in the laboratory (Gray and Cade, 1999). This indicated that gametic disequilibrium and not pleiotropy (or strong physical gene linkage) had caused the correlation.

Given the lack of evidence for genetic coupling, it becomes difficult to explain the evolution of thousands of moth species, and especially evolutionarily closely related species that have unique pheromone blends. A rare female with a mutation leading to an alteration in her pheromone blend is expected to have lower mating success than normal females, unless males fail to discriminate between the typical and altered blends (Butlin and Trickett, 1997). But in all cases studied, normal males do in fact discriminate against females with atypical pheromonal signals (e.g., Zhu et al., 1997). Similarly, a male with a mutation that results in response

to an altered female pheromone blend is expected to be less efficient at finding typical females. Evidence of this lower efficiency comes from studies of moth genotypes that differ in pheromone responses (e.g., Linn et al., 1997). This selection against new male and female mating traits, when they are at low frequency, is expected to constrain the evolutionary diversification of moth sexual communication systems (Butlin and Ritchie, 1989; Phelan, 1997). Recent efforts to resolve this puzzling situation have focused on understanding if the genetic architecture of variation in signal production and response is structured in a way that would allow coevolution of male and female aspects of sexual signaling in insects (see Phelan, 1997).

So far, the genetic architecture of variation in signal and response has been most thoroughly studied in *Ostrinia* (corn borer) species (Lepidoptera: Pyralidae), which have two-component sex pheromone blends. The ratios of the acetate pheromone components (*Z*)-11-tetradecenyl acetate (*Z*11–14:OAc) and *E*11–14:OAc, produced by females of two races of *O. nubilalis* differ dramatically (97:3 vs. 3:97), and are mostly controlled by a single autosomal gene (Klun, 1975; Löfstedt et al., 1989) that is not linked to a second gene that controls male behavioral response to the pheromone (Löfstedt et al., 1989; Cossé et al., 1995; Linn et al., 1999). Studies of $F_2$ and backcross progeny from hybridization of other lepidopteran species have also uncovered evidence for single gene control of production of specific pheromone component ratios (Haynes and Hunt, 1990; Jurenka et al., 1994; Zhu et al., 1997). Recently, intriguing data were published indicating that a single change in a desaturase gene caused appearance of a novel pheromone component within the genus *Ostrinia* (Roelofs and Rooney, 2003).

In many related moth species, blend differences involve multiple components, rather than a simple change in the ratio of two components. So far, few studies have focused on the genetic architecture of variation in multi-component blends. A noteworthy exception is a lab-derived *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) mutant, in which a (single) genetic change in a chain-shortening enzyme was correlated with alteration in multiple component ratios (Haynes and Hunt, 1990; Jurenka et al., 1994; Zhu et al., 1997). Genetic correlations between ratios of multiple components are critical for understanding the evolution of multi-component pheromone blends.

*Heliothis virescens* (Hv) and *H. subflexa* (Hs) (Lepidoptera: Noctuidae) are two closely related moth species with multi-component pheromone blends. The compounds that are emitted from the pheromone gland of Hv females are *Z*11–16:Ald, 14:Ald, *Z*9–14:Ald, 16:Ald, *Z*7–16:Ald, and *Z*9–16:Ald (Pope et al., 1982; Tumlinson et al., 1982; Teal et al., 1986; Heath et al., 1991). The components that are emitted from the pheromone gland of Hs females are *Z*11–16:Ald, 16:Ald, *Z*7–16:OAc, *Z*9–16:OAc, *Z*11–16:OAc, *Z*9–16:OH, and *Z*11–16:OH (Heath et al., 1991). The behavioral role of some of these components is unequivocal. *Z*11–16:Ald is the most abundant sex pheromone component in both species

and it is essential for attraction in both species. *Z*9–14:Ald is also essential for attraction of Hv males, although this component occurs at only ~5% of the amount of *Z*11–16:Ald in Hv pheromone glands (Roelofs et al., 1974; Tumlinson et al., 1975, 1982; Vetter and Baker, 1983; Ramaswamy et al., 1985; Teal et al., 1986; Vickers and Baker, 1997). Hs males are only attracted when both *Z*9–16:Ald and *Z*11–16:OH are present in addition to *Z*11–16:Ald (Teal et al., 1981; Vickers and Baker, 1997; Vickers 2002). Although these minimal blends attract males, blends comprising all emitted components generally increase male attraction in the field. For example, the Hv minimal two-component blend (*Z*11–16:Ald and *Z*9–14:Ald) caught an average of 7.8 Hv males per night, whereas a seven-component blend (adding 14:Ald, 16:Ald, *Z*7–16:Ald, *Z*9–16:Ald, and *Z*11–16:OH) caught 38.1 Hv males per night (Klun et al., 1980). Several studies tested different combinations of pheromone gland components and found antagonistic effects of some pheromone components. For instance, Hs attraction was reduced when *Z*11–16:OH was present in high amounts relative to *Z*11–16:Ald (Teal et al., 1981; Heath et al., 1990), while Hv male attraction was reduced when the heterospecific component *Z*11–16:OAc was added to the minimal two-component pheromone blend (Vickers and Baker, 1997). Components that have been found in pheromone glands but not in volatile blends (i.e., 14:Ald, *Z*9–14:Ald, and 16:OAc in Hs females, and 14:OH, *Z*9–14:OH, and 16:OH in Hv females) are unlikely to play a role in sexual communication, and their presence is probably a consequence

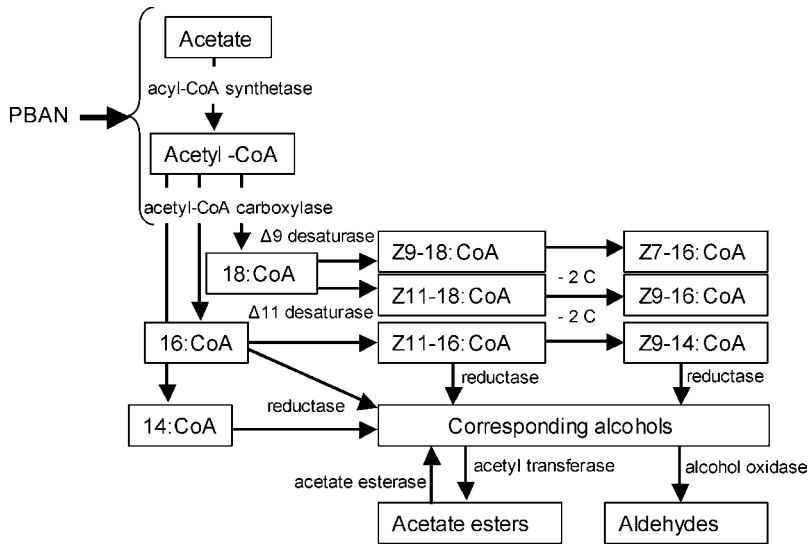


FIG. 1. Generalized biosynthetic pathways of the pheromone components in Hv and Hs. Adapted from Jurenka (2003).

of the biosynthetic pathways of the pheromone components (Figure 1). Because *Z*7–16:OAc and *Z*9–16:OAc have not been tested systematically and independently, their role in attracting Hs males or inhibiting the attraction of Hv males remains unclear.

In a recent study using amplified fragment length polymorphism (AFLP) markers, we found a total of 11 quantitative trait loci (QTL) affecting the amounts of four pheromone components that differ in ratio, or presence/absence, between Hv and Hs (Sheck et al., unpublished). Narrowly defined, a QTL is a single locus that causes a phenotypic effect. However, in practice a QTL is typically defined as a region on a chromosome with little recombination, which contains both a phenotype-altering locus and one or more segments of DNA that can be visualized as markers to indicate the presence of the phenotype-altering locus. In our case, the markers are AFLP fragments, and the region marked is 1 of 31 whole chromosomes because there is no recombination in female Lepidoptera (Robinson, 1971; Turner and Sheppard, 1975; Heckel, 1993; Heckel et al., 1997). In general, Lepidoptera contain 30 autosomes and one sex chromosome, so that each chromosome should, on average, include ~3% of the insect's DNA (Chen and Graves, 1970). The 3% level of QTL resolution is similar to or finer than that in many QTL analyses where recombination is present (e.g., Hawthorne and Via, 2001).

Sheck et al. (unpublished) found the 11 QTL by hybridizing Hv females with Hs males, and subsequently backcrossing $F_1$ females to Hs males, all in single-pair matings. The pheromone gland contents of $BC_1$ (first backcross) females were analyzed by GC, and the presence of Hv chromosomes was determined by AFLP markers, after which the amount of phenotypic variation in the relative amount of pheromone components in $BC_1$ females, that could be explained by presence/absence of one copy of a particular Hv chromosome, was determined. The strongest effects were found for Hv-chromosome 13, explaining 34% of the variance in percentage of 14:Ald, and Hv-chromosome 22, explaining 23% of the variance in percentage of the three acetate esters in the pheromone gland (Sheck et al., unpublished).

Because our long-term goal has been to understand how a single QTL for an alteration in sexual communication can increase in a natural moth population, we wanted to be able to examine the effects of single QTL for sexual communication from one species, when placed in the genomic background of a second, related species. In this paper, we describe how we used a repeated backcrossing procedure to produce hybrid strains that were genetically identical to Hs except for having Hv-chromosome 22. This backcrossing procedure involved a protocol that enabled us to analyze both the pheromone composition and chromosomal make-up of successfully backcrossed, individual moths. We also report data on the pheromone composition of females from these repeated backcrosses that do and do not have a copy of Hv-chromosome 22.

## METHODS AND MATERIALS

*Moth Strains and Backcross Procedures.* In October 2001, 36 single-pair matings were set up, hybridizing *H. virescens* females (YDK strain) with *H. subflexa* males, where each female was identified by using 1–2 letters of the alphabet. The parental strains of Hv and Hs were the same as those described by Sheck et al. (unpublished), and had been in the lab for ~160 and ~40 generations, respectively. Of these 36 single-pair matings, 14 produced at least 30 offspring. Female DD (i.e., the 30th single-pair cross) produced the most offspring, and the female offspring of this cross were backcrossed to Hs males in another set of single-pair matings, to produce $BC_1$. The 23rd daughter of DD produced the most offspring, henceforth called family DD23, which is the backcross line used for the experiments described in this paper (Figure 2). In all subsequent backcrosses, females from previous backcross families were backcrossed to Hs males in single-pair matings, with the aim of isolating Hv-chromosome 22 in the Hs genomic background.

*AFLP Mapping.* DNA from individual grandparents, parents, and offspring was extracted, purified, ligated, and amplified, after which AFLP fragments were separated on the basis of size with a LI-COR sequencer (Lincoln, NE). Our AFLP protocol was adapted from Remington et al. (1999) and Vos et al. (1995), and summarized here. We used the Qiagen Qiamp DNA Mini Kit, mousetail protocol with some modifications. DNA was extracted from half of an adult thorax, which was ~20 mg of tissue. Tissue was incubated overnight at 55°C with 180 $\mu$l lysis buffer and 20 $\mu$l proteinase K per sample, then centrifuged to precipitate the chitin. RNase A (3 $\mu$l @ 4 mg/ml) 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% elution buffer. The final volume was 200 $\mu$l per sample. Samples typically had between 2.4–5 $\mu$g DNA per 20 mg of tissue. For the restriction step, we started with ≤300 ng of genomic DNA. For a total reaction

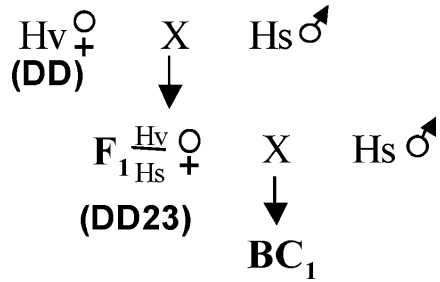


FIG. 2. General mating design for crosses between Hv and Hs, followed by backcrosses to Hs males.

volume of 30.0 $\mu$l, we added 6 U of *Eco*R1, 8 U of *Mse*1, and 6 $\mu$l of 5 $\times$ R/L buffer. The restriction digest incubated for 2 hr at 37°C, after which we stopped the reaction by incubating at 70°C for 15 min. The ligation step started with 20 $\mu$l of restricted DNA. For a total reaction volume of 25 $\mu$l, we added 0.5 $\mu$l of *Eco*R1 adapter (5 pmol/$\mu$l), 0.5 $\mu$l of *Mse*1 adapter (50 pmol/$\mu$l), 0.5 $\mu$l ATP (10 mM), 1 $\mu$l 5 $\times$ R/L buffer, 1/2 U of T4 Ligase. This reaction incubated overnight at 37°C or room temperature. The restricted, ligated DNA (R/L DNA) was diluted 1:10. The pre-amplification step was started with 5 $\mu$l of the diluted R/L DNA, to which 20.0 $\mu$l of preamp mix I (Gibco/BRL), 2.5 $\mu$l of 10 $\times$ PCR buffer+Mg (Boerhringer Mannheim), and 2.5 U of *Taq* polymerase were added. We used the following PCR amplification profile: 28 cycles, 30 sec @ 94°C, 30 sec @ 60°C, 60 sec @ 72°C. The pre-amplified DNA was diluted 1:40 by transferring 24 $\mu$l of the reaction product to a deep-well plate and adding 936 $\mu$l sd$H_2O$. Selective amplification used 3 $\mu$l of pre-amplified R/L DNA. For a total reaction volume of 12 $\mu$l, we added 3 $\mu$l of M primer (6 ng/$\mu$l), 1.2 $\mu$l of 10 $\times$ PCR buffer, 0.48 $\mu$l of dNTP, 5 mM, 0.14 $\mu$l of *Taq* polymerase (5 U/$\mu$l), 0.5 $\mu$l of IRD labeled E primer (LiCor), and sd$H_2O$ to bring up the volume. The core sequence of the E primer was 5′-GAC TGC GTA CCA ATT C, and the core sequence of the M primer was 5′-GAT GAG TCC TGA GTA A. We added three selective bases to the end of each primer (Sheck et al., unpublished). The PCR amplification profile was as follows: cycles 1–13: 10 sec @ 94°C, 30 sec @ 65°C; −0.7°C/cycle; 60 sec @ 72°C; cycles 14–36: 10 sec @ 94°C; 30 sec @ 56°C; 60 sec @ 72°C+1 sec/cycle. AFLP fragments were separated based on size with a LI-COR 4200 sequencer that, with a scanning laser, simultaneously detects infared labeled DNA fragments of 700 and 800 nm. The samples were prepared for 8% polyacrylamide gels by adding 6 $\mu$l of formamide loading dye [95% formamide, 20 mM EDTA, bromophenol blue (USB)] per 12 $\mu$l reaction. Samples were denatured at 90°C for 3 min and immediately placed on ice. Samples were loaded into 96 wells (0.7–1.0 $\mu$l per well) with a Hamilton syringe. A labeled standard (LI-COR STR marker, 50–700 bp) was loaded at each end of the gel. The original parents and $F_1$ cross were always loaded into wells two to five. The gels were run for about 3.5 hr, and the images were recorded in a computer file. We scored the gels with a semiautomatic image analysis program designed specifically for AFLP analysis (Quantar 1.08, KeyGene Products).

The markers of interest were those that were present as bands in gels from the Hv parent and the $F_1$ female, but absent in the original Hs parent or the recurrent Hs backcross parent. For the construction of the DD23 linkage map, we used 88 $BC_1$ individuals (both males and females), and a total of 14 primer pairs. In order to maximize our chance of finding similar AFLP markers that identify the 31 chromosomes in the different backcross families, we used primer pairs that had previously been used to map the backcross families in which the major effect of chromosome 22 on the production

of acetates was found (i.e., families C5 and C6). In backcrosses subsequent to $BC_1$, we analyzed females for the presence of Hv-chromosome 22 on the basis of a subset of four AFLP markers that identified Hv-chromosome 22 in DD23.

*Pheromone Analysis.* Female moths typically cease pheromone production after they have mated. This posed a problem for us, because we needed to determine the pheromone gland content of BC females *after* they had mated and oviposited in order to continue the lines. Therefore, we developed a procedure based on pheromone biosynthesis activating neuropeptide (PBAN) injections that caused mated BC females to produce their normal, genetically determined pheromone blend after ovipositing fertile eggs (Groot et al., 2004). This method was developed subsequent to our finding that the majority of the pheromone glands of $BC_1$ and $BC_2$ females had only trace amounts of pheromone compounds after mating and oviposition. The glands of subsequent backcross females were extracted and analyzed as follows (see Groot et al., 2004, for additional details). Females were injected during the photophase with 1 pmol *Hez*-PBAN (Peninsula Laboratories, San Carlos, CA) in 1 $\mu$l saline, using a 10 $\mu$l syringe with a 31 gage needle (Hamilton, Reno, NV) that was inserted ventrally between the 8th and the 9th abdominal segments. One hour after injection, the pheromone glands were dissected and extracted in conical vials containing 50 $\mu$l 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 the extract was stored at $-20^{\circ}$C until analysis. The hexane extracts were reduced to 0.5–1.5 $\mu$l under a gentle stream of $N_2$, and each sample was then injected into a pulsed splitless injector held at 240°C in an HP6890 GC, and separated using a 30 m $\times$ 0.25 mm ID $\times$ 0.5 $\mu$m film thickness Stabilwax column (Restek, Bellefonte, PA) temperature programed from 60°C (2 min) to 180°C at 30°C/min, then to 230°C at 2°C/min, during which all the pheromone components eluted. The carrier gas was helium at 30 cm/sec, and the FID was held at 240°C. The amount of each pheromone component was calculated relative to the 20 ng of internal standard. The pheromone components that we distinguished were: 14:Ald, *Z*9–14:Ald, 16:Ald, *Z*7–16:Ald, *Z*9–16:Ald, *Z*11–16:Ald, *Z*7–16:OAc, *Z*9–16:OAc, *Z*11–16:OAc, and *Z*11–16:OH. Most chromatographic analyses did not separate *Z*7–16:Ald from *Z*9–16:Ald. Even though *Z*7–16:Ald is present only in low amounts in both species, and *Z*9–16:Ald is present in relatively large amounts in Hs, we combined the peak areas of these two compounds and denoted the combination as *Z*7/9–16:Ald. The proportions of all pheromone components were calculated as relative percentages of the total amount of pheromone, so that the pheromone composition could be compared among females.

*Isolation of Hv-Chromosome 22.* After pheromone gland extraction, the backcross females were frozen at $-80^{\circ}$C. $BC_3$, $BC_4$, and $BC_9$ females were genotyped with the primer pairs that identified all Hv-chromosomes in DD23 to

determine the presence of any Hv-chromosomes. Selection for Hv-chromosome 22 started with $BC_3$ females. All females that had Hv-chromosome 22 and a low percentage of acetate esters (i.e., <3% of the total amount of pheromone components present) were backcrossed to Hs males. In $BC_5$, $BC_6$, $BC_7$, and $BC_8$, we determined whether Hv-chromosome 22 was present a) by running only the primer pairs that would yield the markers for that chromosome and/or b) by determining the relative amount of acetate esters in the pheromone glands. Either genotype or phenotype, or both were used as a criterion to continue to backcross these females to Hs males. In order to completely isolate Hv-chromosome 22, in $BC_9$ we crossed males to Hs females to eliminate the sex chromosome, as well as mitochondrial DNA, which was inherited from the original Hv female. In the female progeny of this cross, we found that all AFLP markers that identify Hv-chromosome 22 were present, indicating that no recombination had occurred in this chromosome. Backcrosses were only continued with females in which all Hv-chromosome 22 markers were present.

*Effect of Hv-Chromosome 22 on Pheromone Composition.* In $BC_3$ to $BC_5$, and in $BC_{11}$ and $BC_{12}$, we genetically verified the presence of Hv-chromosome 22, and we analyzed the pheromone composition of all females. ANOVA [PROC GLM in SAS, version 8.02 (SAS Institute, 2002)] was used to assess the effect of presence or absence of Hv-chromosome 22 (i.e., genotype) on the relative amount of the different pheromone components (i.e., phenotype). After $BC_9$, the only genomic Hv chromosome in any BC female was Hv-chromosome 22. We determined whether the variation in pheromone composition in $BC_{11}$ due to presence or absence of Hv-chromosome 22 was similar to the variation in pheromone composition in $BC_3$ and $BC_5$, by comparing the variation of each pheromone component in females with Hv-chromosome 22 between these three backcrosses. The $R^2$ value from the ANOVA provided an estimate of how much of the phenotypic variation in the production of all pheromone components was explained by the presence or absence of one copy of Hv-chromosome 22.

## RESULTS

*AFLP Mapping.* The primer pairs that we used produced a total of 215 AFLP markers, 193 of which were grouped into 31 linkage groups in the mapping program Mapmaker (version 3, 1993, Whitehead Institute for Biomedical Research). Because there was no recombination, each linkage group represented a whole chromosome. Since we used both males and females, we could easily distinguish the sex chromosome by determining which of the grouped markers were present in females and absent in males. This analysis indicated that linkage group 23 was the sex chromosome. The average number of markers per chromosome was 6.23, with one chromosome (31) containing two markers from

TABLE 1. MEAN AMOUNT OF PHEROMONE IN GLANDS OF MATED, >4-DAY-OLD, BACKCROSS FEMALES INJECTED WITH 1 PMOL *Hez*-PBAN

| | $BC_3$ | $BC_4$ | $BC_5$ | $BC_6$ | $BC_{10}$ | $BC_{11}$ | $BC_{12}$ |
|---|---|---|---|---|---|---|---|
| Amount (ng ± SEM) | 293 ± 22 | 293 ± 19 | 360 ± 18 | 270 ± 9 | 187 ± 22 | 280 ± 8 | 250 ± 15 |
| *N* females analyzed | 41 | 98 | 147 | 133 | 31 | 205 | 79 |
| *N* with <50 ng[a] | 1 | 3 | 0 | 3 | 2 | 2 | 3 |

[a]Females with <50 ng total pheromone.

different primer pairs, one containing three markers, two containing four markers, and the rest containing at least five markers. For chromosome 22, we found one informative marker (223 bp in length, with E-primer AGC and M-primer CTT) that was also present in families C5 and C6 (Sheck et al., unpublished). To confirm the chromosomal homology between families DD23, C5, and C6, we alternated slots on one polyacrylamide gel with C5 individuals having chromosome 22, and C5 individuals lacking chromosome 22, which was repeated for C6 and DD23 individuals. All individuals were chosen based on similarity in presence of other chromosomes, a requirement for finding markers specifically for chromosome 22. Using this procedure we found one other marker that was present in all three groups that had Hv-chromosome 22 (120 bp in length, with E-primer AAC and M-primer CTC). These markers grouped together with eight other DD23-specific markers when using Mapmaker, thus identifying chromosome 22 in DD23.

*Pheromone Analysis*. Since a number of pheromone components in Hv and Hs are <1% of the total pheromone blend, we found it impossible to accurately determine the relative amount of each component when the gland contained <50 ng of pheromone, as was common in mated females. However, injection of mated, >4-day-old, backcross females with 1 pmol PBAN yielded 187–360 ng of total pheromone in each gland (Table 1). Of the total of 734 BC females from which glands were extracted after PBAN injection, only 14 contained <50 ng pheromone (Table 1). These females were excluded from further analysis.

*Isolation of Hv-Chromosome 22*. In general, each backcross will result in the loss of (on average) half the chromosomes of the non-recurrent parent, in this case Hv (Figure 3). $BC_3$ females with Hv-chromosome 22 contained some of the other Hv chromosomes as well (Table 2). However, none of the other Hv chromosomes that were found in these BC females showed a significant effect on the variation in acetate pheromone production (results not shown). Selecting $BC_3$ females with Hv-chromosome 22 for subsequent backcrossing resulted in 16 out of 29 (55%) of offspring having Hv-chromosome 22 in $BC_4$ (Table 3). When $BC_4$ females with Hv-chromosome 22 were backcrossed to Hs males, 39 out of 74 (53%) offspring had Hv-chromosome 22 (Table 3).

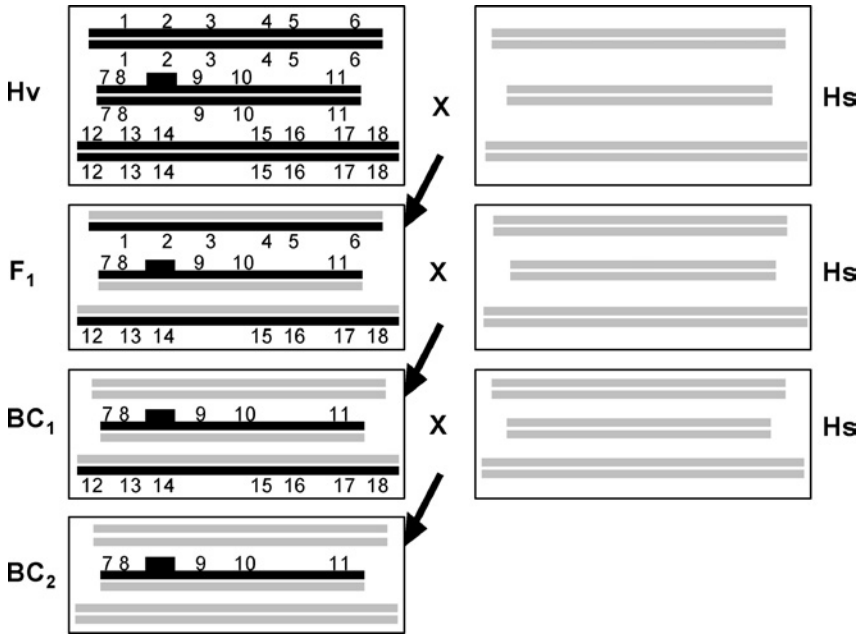


FIG. 3. General schematic example of how recurrent backcrossing with selection (and no recombination in hybrid females) can result in isolation of one Hv chromosome in an Hs genomic background. For simplicity only three pairs of chromosomes, two backcrosses (BC), and one class of the segregating genotypes are shown. Chromosomes from Hv are black, those from Hs are gray. Short black rectangle represents allele coding for pheromone component. Each number represents an AFLP marker mapped to a chromosome.

Backcross females with Hv-chromosome 22 consistently contained a total of $<3\%$ of the three acetate pheromone components in their gland, whereas backcross females without Hv-chromosome 22 typically had $>3\%$ (Figure 4B–D). Hs females and Hv females contained $16.56\% \pm 1.51\%$ (mean $\pm$ SEM) and 0% total acetates, respectively (Figure 4). Therefore, the 3% level was used as a phenotypic threshold for determining the presence or absence of Hv-chromosome 22 up to $BC_{12}$(Table 4). From $BC_{12}$ on, the total percentage of acetates slightly increased when Hv-chromosome 22 was present, but was never $>5\%$ of the total pheromone content of the gland. In subsequent backcrosses, the percentage of acetates was always contrastingly higher in BC females without Hv-chromosome 22, typically between 10% and 15%, than in BC females with Hv-chromosome 22 (results not shown).

In $BC_4$ females with Hv-chromosome 22, the total number of Hv-autosomes represented in at least one of the 12 females was reduced to 8 (compared to 19 in

TABLE 2. PRESENCE OF OTHER HV-CHROMOSOMES IN FEMALES FROM THE THIRD, FOURTH, AND NINTH BACKCROSSES

| | Number of BC females with Hv-chromosomes | | | | |
|---|---|---|---|---|---|
| Hv-chromosome present | In $BC_3$ with Hv-chromosome 22 ($N = 14$) | In $BC_3$ all females ($N = 35$) | In $BC_4$ with Hv-chromosome 22 ($N = 12$) | In $BC_4$ all females ($N = 42$) | In $BC_9$ ($N = 7$) |
| C01 | 0 | 0 | 0 | 0 | 0 |
| C02 | 3 | 5 | 5 | 2 | 0 |
| C03 | 1 | 4 | 0 | 0 | 0 |
| C04 | 3 | 7 | 2 | 8 | 0 |
| C05 | 2 | 7 | 0 | 2 | 0 |
| C06 | 0 | 0 | 0 | 0 | 0 |
| C07 | 5 | 7 | 2 | 4 | 0 |
| C08 | 0 | 2 | 0 | 0 | 0 |
| C09 | 0 | 0 | 0 | 0 | 0 |
| C10 | 2 | 4 | 0 | 0 | 0 |
| C11 | 0 | 0 | 0 | 0 | 0 |
| C12 | 1 | 2 | 0 | 2 | 0 |
| C13 | 0 | 0 | 0 | 0 | 0 |
| C14 | 1 | 1 | 0 | 5 | 0 |
| C15 | 0 | 0 | 0 | 0 | 0 |
| C16 | 0 | 0 | 0 | 0 | 0 |
| C17 | 0 | 1 | 0 | 1 | 0 |
| C18 | 5 | 9 | 0 | 0 | 0 |
| C19 | 2 | 4 | 4 | 11 | 0 |
| C20 | 5 | 9 | 2 | 9 | 0 |
| C21 | 3 | 13 | 0 | 0 | 0 |
| C22 | 14 | 14 | 12 | 12 | 7 |
| C23 (sex chr.) | 14 | 35 | 12 | 42 | 3[a] |
| C24 | 0 | 0 | 0 | 0 | 0 |
| C25 | 6 | 12 | 1 | 7 | 0 |
| C26 | 2 | 4 | 1 | 3 | 0 |
| C27 | 1 | 1 | 0 | 1 | 0 |
| C28 | 2 | 2 | 0 | 5 | 0 |
| C29 | 3 | 7 | 0 | 0 | 0 |
| C30 | 4 | 6 | 0 | 5 | 0 |
| C31 | 6 | 10 | 3 | 8 | 0 |

[a] In $BC_9$, we backcrossed four males to Hs-females to delete the Hv-sex chromosome. The family that retained all Hv-chromosome 22 markers (i.e., family 50-210-46-46-60-712-1716-2625-300) was continued into $BC_{10}$.

14 $BC_3$ females) (Table 2). In $BC_9$ females, the Hv-sex chromosome was the only Hv-chromosome present in addition to chromosome 22. Backcrossing $BC_9$ males deleted the Hv-sex chromosome, and in one female offspring, all Hv-chromosome 22 markers were retained, indicating that no recombination had occurred in that family. Subsequent backcrosses were conducted with that female.

TABLE 3. INHERITANCE OF HV-CHROMOSOME 22 WITH SELECTION

| $BC_3$-mother[a] with Hv-chromosome 22 | $BC_4$ offspring with Hv-chromosome 22 | $BC_4$ mother* with Hv-chromosome 22 | $BC_5$ offspring with Hv-chromosome 22 |
|---|---|---|---|
| 4-114-24 | 3 of 4 | 4-114-24-63 | 2 of 3 |
| 4-114-34 | 2 of 3 | 4-114-34-1 | 20 of 37 |
| 4-114-44 | 2 of 3 | | |
| 50-197-9 | 5 of 9 | 50-197-9-207 | 1 of 2 |
| | | 50-197-7-208 | 7 of 12 |
| 50-210-46 | 1 of 6 | 50-210-46-46 | 1 of 1 |
| 50-210-503 | 3 of 4 | 50-210-503-203 | 8 of 19 |

[a]Name of the mother, designated by a specific number in each generation to keep track of each specific family line.

*Effect of Hv-Chromosome 22 in Isolation*. In $BC_3$ females, the percentage of *Z*7-16:OAc was marginally significantly different between females with and females without Hv-chromosome 22 (means ± SEM are 0.43 ± 0.12 and 0.16 ± 0.09, respectively, $P = 0.06$) (Figure 4B). However, the percentages of *Z*9–16:OAc (2.46 ± 0.66 in females without Hv-chromosome 22, and 0.26 ± 0.1 in females with Hv-chromosome 22, $P = 0.006$) and *Z*11–16:OAc (6.94 ± 1.58 in females without Hv-chromosome 22 and 1.28 ± 0.41 in females with Hv-chromosome 22, $P = 0.004$) were significantly different. Also, the cumulative percentage of all acetates was different between $BC_3$ females without Hv-chromosome 22 and females with Hv-chromosome 22 (9.83 ± 2.31 and 1.69 ± 0.55, respectively, $P = 0.004$). The $R^2$ value of the latter comparison was 0.20, indicating that 20% of the phenotypic variation in the production of the acetates was explained by the presence/absence of one copy of Hv-chromosome 22.

In $BC_5$ and $BC_{11}$, the percentages of all three acetates were different ($P < 0.001$ in both BC's) between females with and without Hv-chromosome 22 (Figure 4C and D). For all three acetates combined, the $R^2$ value in $BC_5$ was 0.31, while in $BC_{11}$ it was 0.51. In $BC_5$ females, *Z*9–14:Ald was also significantly different between females with and without Hv-chromosome 22; females with Hv-chromosome 22 had 0.51% ± 0.06% of Z9-14:Ald, whereas in females without the Hv-chromosome *Z*9–14:Ald represented 0.37% ± 0.04% of the total amount of pheromone ($P = 0.044$, $R^2 = 0.068$). In $BC_{11}$ females, 14:Ald and *Z*11–16:Ald showed differences between the two groups, in addition to the highly significant differences in acetates ($P < 0.001$ for all three acetates). The mean percentage of 14:Ald was 0.35 ± 0.01 in females with, and 0.31 ± 0.01 in females without Hv-chromosome 22 ($P = 0.023$, $R^2 = 0.035$). The mean percentage of *Z*11–16:Ald was 51.1 ± 0.84 in females with, and 47.94±0.84 in females without Hv-chromosome 22 ($P = 0.009$, $R^2 = 0.045$).

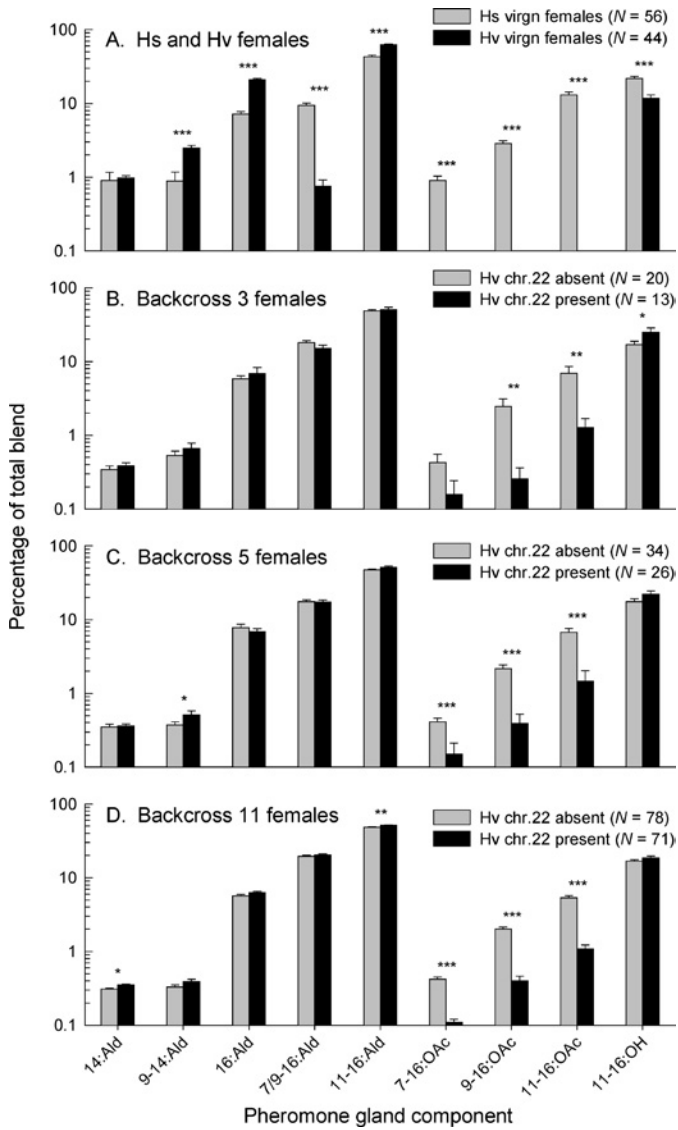


FIG. 4. Pheromone profiles of virgin Hv and Hs females (A), and of $BC_3$, $BC_5$, and $BC_{11}$ females (B–D) with and without Hv-chromosome 22 as determined by AFLP analysis. The backcross females were injected with 1 pmol *Hez*-PBAN before glands were extracted. Percentages of pheromone components are graphed on a logarithmic scale to highlight differences between secondary components. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. When no asterisk is shown, differences are not significant.

TABLE 4. ABILITY TO PREDICT GENOTYPE FROM PHENOTYPE

| | % Females with >3% acetates (Hv-chromosome 22 absent) or <3% acetates (Hv-chromosome 22 present)[a] (*N*) | | | | |
|---|---|---|---|---|---|
| Hv-chromosome 22 | $BC_3$ | $BC_4$ | $BC_5$ | $BC_{11}$ | $BC_{12}$ |
| Absent and >3% | 85 (20) | 95 (39) | 80 (34) | 90 (74) | 95 (19) |
| Present and <3% | 77 (13) | 92 (13) | 71 (34) | 93 (69) | 67 (21) |

[a] Percentages of females with Hv-chromosome 22 absent that had a total percentage of acetates >3% of the total pheromone blend, and females with Hv-chromosome 22 present in which the total percentage of acetates was <3% of the total pheromone blend.

## DISCUSSION

By using phenotypic as well as genotypic (marker-specific) traits, we have successfully isolated Hv-chromosome 22 in an Hs nuclear and mitochondrial genomic background. Although Hv females do not contain any acetate esters in their pheromone gland, BC females with a single copy of Hv-chromosome 22 generally contained a small amount of acetate components, which usually summed to <3% of the total pheromone blend. The fact that acetate production is not completely shut down in females with Hv-chromosome 22 suggests that the heterozygous presence of this chromosome is not enough to completely inhibit acetate accumulation in the glands. The absence of acetates in Hv female glands could be due to more than one specific step in the biosynthetic pathway (Bjostad and Roelofs, 1983; Morse and Meighen, 1986; Teal and Tumlinson, 1987). In general, the acetates are thought to be synthesized from the corresponding alcohols through acetyl transferase (Figure 1), and a difference between Hv and Hs in presence or activity of this enzyme could explain the different amounts of acetate esters in their respective pheromone blends. However, acetate esters can be converted back to the corresponding alcohols through acetate esterase (Figure 1). Teal et al. (1989) found significantly higher acetate esterase activity in Hv pheromone glands, suggesting that any acetate esters produced by Hv would be immediately converted back to the corresponding alcohols. Thus, an alternative explanation for the differences between Hv and Hs might involve the presence or activity of acetate esterase.

In $BC_3$ females, the percentage of *Z*11–16:OH significantly correlated with presence of Hv-chromosome 22, in addition to the percentages of the acetate esters. A similar result has been found in the previous C5 and C6 $BC_1$ females, and matches with the negative phenotypic correlation between *Z*11–16:OH and each of the three acetates (Sheck et al., unpublished). It could be that the factor associated with chromosome 22 contributes to conversion of acetates to alcohols (acetate esterase). Alternatively, there may be separate QTL on chromosome 22 that control acetate and alcohol production. In $BC_5$ and $BC_{11}$ females, the correlation between *Z*11–16:OH and Hv-chromosome 22 was lost. This suggests that presence of

Hv-chromosome 22 alone in an Hs genetic background is not sufficient to affect *Z*11–16:OH levels. It may be that QTL on Hv-chromosome 22 interacts epistatically with QTL on other Hv chromosomes to affect the level of this alcohol.

In $BC_5$ females, *Z*9–14:Ald was significantly correlated with the presence of Hv-chromosome 22, while in $BC_{11}$ females, 14:Ald and *Z*11-16:Ald showed significant differences between females with and females without Hv-chromosome 22. However, the statistically significant differences in the relative amounts of 14:Ald and *Z*9–14:Ald may not be biologically significant, because the differences in the quantities produced were very small. Previously, we confirmed the presence of small amounts of these components in Hs females (Groot et al., 2004). The percentage of 14:Ald relative to the main component was similar in virgin Hs and virgin Hv females (Figure 4A), suggesting that the significant differences in $BC_{11}$ may indeed be an artifact due to the low amount of 14:Ald. The observation that the major component, *Z*11–16:Ald, was significantly higher in $BC_{11}$ females with than in females without Hv-chromosome 22, is likely due to the interdependence of the different components. Since all other components are similar between the two groups, and females without Hv-chromosome 22 contain significantly higher amounts of acetates, the percentage of the major component is likely to be lower in those females based on carbon flow in the biosynthetic pathway (Figure 1). The $R^2$ value was very low, only 4.5% of the phenotypic variation in the production of Z11–16:Ald was explained by the presence/absence of one copy of Hv-chromosome 22, while 51% of the phenotypic variation in the production of the acetates was explained by the presence/absence of this chromosome.

The effect of Hv-chromosome 22 on the variation in pheromone composition is only the first step toward our long-term goal of understanding the evolutionary processes that have resulted in diversification of moth sexual communication signals and responses. Currently, we are conducting cage and field experiments with the introgressed lines described here to determine how the difference in this chromosome, and the corresponding reduction of acetate production, affect the likelihood of a female being mated by an Hv or Hs male. So far, acetates have not been rigorously demonstrated to function as attractants for Hs males (Teal et al., 1981; Tumlinson et al., 1982; Vickers, 2002), but Vickers and Baker (1997) have shown that *Z*11–16:OAc deter Hv males. The simplest evolutionary scenario for evolution of presence of acetates would be that deterring heterospecific males would have been a driving force for a shift in the pheromone blend to which acetates were added. Now that we have succeeded in isolating the chromosome that has a major effect on acetate production, we can test this hypothesis through behavioral experiments. Such tests are necessary to move towards an evolutionary understanding of the differentiation in sexual communication in *Heliothis* spp. moths with multi-component sex pheromone blends.

Fine-scale mapping and cloning of the QTL can help explain which gene(s) are involved in control of acetate production, and how they interact biochemically.

Currently, we are saturating the chromosome with as many AFLP markers as we can (we now have 18 markers; J. L. Bennett, unpublished results), and we are conducting backcrosses in which the Hs parent is the female and the male parent is from the introgression line carrying the Hv-chromosome 22. Because there is crossing-over in male chromosomes, this type of cross allows recombination between segments of Hv- and Hs-chromosome 22. The offspring of these crosses will be used for developing lines that are homozygous for only a small segment of Hv-chromosome 22 that contains the QTL for acetate levels. Also, specific differences in the biochemical pathways of the pheromone components between Hv and Hs are currently being unraveled (Choi et al., 2004), which can lead to the identification of candidate genes. Earlier we have demonstrated that it was possible to use a combination of mapping and candidate genes to identify and sequence one important gene in Hv (Gahan et al., 2001). With the identification of genes underlying pheromonal differences between the species, the sequences of these genes in different geographic populations can be examined to determine patterns of synonymous and non-synonymous substitutions within and between species, indicating the action of selection (e.g., Taylor et al., 1996; Tsaur et al., 1998). The patterns of allelic substitution and diversity in these species will provide insights about which substitutions were critical in the diversification of the signal/response system (e.g., Saez et al., 2003).

*Acknowledgments*—We thank Cavell Brownie and Keith Hopper for help with statistical analyses, Peter Teal and Sonny Ramaswamy for gifts of synthetic compounds, and Seth Baines, Chang Su, Maude Rowland, Kate Dulaney, Natalie Taylor, Jaclynn Szwagiel, Thembekile Dube, Dorit Eliyahu, and Yongliang Fan for technical help. This research was supported in part by grants from NSF Population Biology 0235400 and the W. M. Keck Center for Behavioral Biology, and by the Blanton J. Whitmire Endowment at North Carolina State University.

## REFERENCES

BJOSTAD, L. B. and ROELOFS, W. L. 1983. Sex pheromone biosynthesis in *Trichoplusia ni*: Key steps involve delta-11 desaturation and chain-shortening. *Science* 220:1387–1389.

BUTLIN, R. 1995. Genetic variation in mating signals and responses, pp. 327–366, *in* D. M. Lambert and H. G. Spencer (eds.). Speciation and the Recognition Concept: Theory and Application. Johns Hopkins Univ. Press, Baltimore.

BUTLIN, R. and RITCHIE, M. G. 1989. Genetic coupling in mate recognition systems: What is the evidence? *Biol. J. Linn. Soc.* 37:237–246.

BUTLIN, R. and TRICKETT, A. J. 1997. Can population genetic simulations help to interpret pheromone evolution? pp. 548–562, *in* R. T. Cardé and A. K. Minks (eds.). Insect Pheromone Research: New Directions. Chapman & Hall, New York.

CHEN, G. T. and GRAVES, J. B. 1970. Spermatogenesis of the tobacco budworm. *Ann. Entomol. Soc. Am.* 63:1095–1104.

COSSÉ, A. A., CAMPBELL, M., GLOVER, T. J., LINN, C. E., JR., TODD, J. L., BAKER, T. C., and ROELOFS, W. L. 1995. Pheromone behavioral responses in unusual male European corn borer

hybrid progeny not correlated to electrophysiological phenotypes of their pheromone-specific antennal neurons. *Experientia* 51:809–816.

COYNE, J. A. and ORR, H. A. 1998. The evolutionary genetics of speciation. *Phil. Trans. R. Soc. Lond. Ser. B: Biol. Sci.* 353:287–305.

GAHAN, L. J., GOULD, F., and HECKEL, D. G. 2001. Identification of a gene associated with Bt resistance in *Heliothis virescens*. *Science* 293:857–860.

GRAY, D. A. and CADE, W. H. 1999. Quantitative genetics of sexual selection in the field cricket, *Gryllus integer*. *Evol.* 53:848–854.

GROOT, A., FAN, Y., BROWNIE, C., JURENKA, R. A., GOULD, F., and SCHAL, C. 2004. Effect of PBAN on the sex pheromone gland profile in mated *Heliothis virescens* and *Heliothis subflexa* females. *J. Chem. Ecol.* (in press).

HAWTHORNE, D. J. and VIA, S. 2001. Genetic linkage of ecological specialization and reproductive isolation in pea aphids. *Nature* 412:904–907.

HAYNES, K. F. and HUNT, R. E. 1990. A mutation in the pheromonal communication system of the cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:1249–1257.

HEATH, R. R., MITCHELL, E. R., and CIBRIAN TOVAR, J. 1990. Effect of release rate and ratio of (*Z*)-11-hexadecen-1-ol from synthetic pheromone blends on trap capture of *Heliothis subflexa* (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 16:1259–1268.

HEATH, R. R., MCLAUGHLIN, J. R., PROSHOLT, F., and TEAL, P. E. A. 1991. Periodicity of female sex pheromone titer and release in *Heliothis subflexa* and *H. virescens* (Lepidoptera: Noctuidae). *Ann. Ent. Soc. Am.* 84:182–189.

HECKEL, D. G. 1993. Comparative genetic linkage mapping in insects. *Annu. Rev. Entomol.* 38:381–408.

HECKEL, D. G., GAHAN, L. J., GOULD, F., and ANDERSON, A. 1997. Identification of a linkage group with a major effect on resistance to *Bacillus thuringiensis* Cry1Ac endotoxin in the tobacco budworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 90:75–86.

HOY, R. R., HAHN, J., and. PAUL, R. C. 1977. Hybrid cricket auditory behavior: evidence for genetic coupling in animal communication. *Science* 195:82–84.

JURENKA, R. A. 2003. Biochemistry of female moth sex pheromones, pp. 53–80, *in* G. J. Blomquist and R. Vogt (eds.). Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, Amsterdam.

JURENKA, R. A., HAYNES, K. F., ADLOF, R. O., BENGTSSON, M., and ROELOFS, W. L. 1994. Sex pheromone component ratio in the cabbage looper moth altered by a mutation affecting the fatty acid chain-shortening reactions in the pheromone biosynthetic pathway. *Insect Biochem. Molec. Biol.* 24:373–381.

KLUN, J. A. 1975. Insect sex pheromones: intraspecific pheromonal variability of *Ostrinia nubilalis* in North America and Europe. *Environ. Entomol.* 4:891–894.

KLUN, J. A., BIERL-LEONHARDT, B. A., PLIMMER, J. R., SPARKS, A. N., PRIMIANI, M., CHAPMAN, O. L., LEPONE, G., and LEE, G. H. 1980. Sex pheromone chemistry of the female tobacco budworm moth *Heliothis virescens*. *J. Chem. Ecol.* 6:177–183.

LINN, C. E., JR. and ROELOFS, W. L. 1995. Pheromone communication in moths and its role in the speciation process, pp. 263–300, *in* D. M. Lambert and H. G. Spencer (eds.). Speciation and the Recognition Concept: Theory and Application. Johns Hopkins Univ. Press, Baltimore.

LINN, C., JR., POOLE, K., ZHANG, A., and ROELOFS, W. L. 1999. Pheromone-blend discrimination by European corn borer moths with inter-race and inter-sex antennal transplants. *J. Comp. Physiol. A* 184:273–278.

LINN, C. E., JR., YOUNG, M. S., GENDLE, M., GLOVER, T. J., and ROELOFS, W. L. 1997. Sex pheromone blend discrimination in two races and hybrids of the European corn borer moth, *Ostrinia nubilalis*. *Physiol. Entomol.* 22:212–223.

LÖFSTEDT, C. 1990. Population variation and genetic control of pheromone communication systems in moths. *Entomol. Exp. Appl.* 54:199–218.

LÖFSTEDT, C. 1993. Moth pheromone genetics and evolution. *Philos. Trans. R. Soc. Lond. Ser. B: Biol. Sci.* 340:167–177.

LÖFSTEDT, C., HANSSON, B. S., ROELOFS, W. L., and BENGTSSON, B. O. 1989. No linkage between genes controlling female pheromone production and male pheromone response in the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae). *Genetics* 123:553–556.

MORSE, D. and MEIGHEN, E. 1986. Pheromone biosynthesis and role of functional groups in pheromone specificity. *J. Chem. Ecol.* 12:335–351.

PHELAN, P. L. 1997. Evolution of mate signalling in moths: Phylogenetic considerations and predictions from the asymmetric tracking hypothesis, pp. 240–256, *in* J. Choe and B. Crespi (eds.). Evolution of MatingSystems in Insects and Arachnids. Cambridge University Press, Cambridge.

POPE, M. M., GASTON, L. K., and BAKER, T. C. 1982. Composition, quantification, and periodicity of sex pheromone gland volatiles from individual *Heliothis virescens* females. *J. Chem. Ecol.* 8:1043–1055.

RAMASWAMY, S. B., RANDLE, S. A., and MA, W. K. 1985. Field evaluation of the sex pheromone components of *Heliothis virescens* (Lepidoptera: Noctuidae) in cone traps. *Environ. Entomol.* 14:293–296.

REMINGTON, D. L., WHETTEN, R. W., LIU, B.-H., and O'MALLEY, D. M. 1999. Construction of an AFLP genetic map with nearly complete genome coverage in *Pinus taeda*. *Theor. Appl. Genet.* 98:1279–1292.

ROBINSON, R. 1971. Lepidoptera Genetics. Pergamon Press, Oxford.

ROELOFS, W. L. and ROONEY, A. P. 2003. Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proc. Natl Acad. Sci. USA* 100:9179–9184.

ROELOFS, W. L., HILL, A. S., CARDÉ, R. T., and BAKER, T. C. 1974. Two sex pheromone components of the tobacco budworm moth, *Heliothis virescens*. *Life Science* 14:1555–1562.

SAEZ, A. G., TATARENKOV, A., BARRIO, E., BECERRA, N. H., and AYALA, F. J. 2003. Patterns of DNA sequence polymorphism at *Sod* vicinities in *Drosophila melanogaster*: Unraveling the footprint of a recent selective sweep. *Proc. Natl Acad. Sci. USA* 100:1793–1798.

TAYLOR, M. F. J., PARK, Y., and SHEN, Y. 1996. Molecular population genetics of sodium channel and juvenile hormone esterase markers in relation to pyrethroid resistance in *Heliothis virescens* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am.* 89:728–738.

TEAL, P. E. A. and TUMLINSON, J. H. 1987. The role of alcohols in pheromone biosynthesis by two noctuid moths that use acetate pheromone components. *Arch. Insect Biochem. Physiol.* 4:261–269.

TEAL, P. E. A., HEATH, R. R., TUMLINSON, J. H., and MCLAUGHLIN, J. R. 1981. Identification of sex pheromone of *Heliothis subflexa* (G.) (Lepidoptera: Noctuidae) and field trapping studies using different blends of components. *J. Chem. Ecol.* 7:1011–1022.

TEAL, P. E. A., TUMLINSON, J. H., and HEATH, R. R. 1986. Chemical and behavioral analyses of volatile sex pheromone components released by calling *Heliothis virescens* (F.) females (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 12:107–125.

TEAL, P. E. A., TUMLINSON, J. H., and OOSTENDORP, A. 1989. Enzyme-catalyzed pheromone synthesis in *Heliothis* moths, pp 332-343, *in* J. R. Whitaker and P. E. Sonnet (eds.). Biocatalysis in Agricultural Biotechnology. ACS Symposium Series 389, American Chemical Society, Washington, DC.

TSAUR, S. C., TING, C. T., and WU, C. I. 1998. Positive selection driving the evolution of a gene of male reproduction, Acp26Aa, of *Drosophila*: II. Divergence vs. polymorphism. *Molec. Biol. Evol.* 15:1040–1046.

TUMLINSON, J. H., HENDRICKS, P. E., MITCHELL, E. R., DOOLITTLE, R. E., and BRENNAN, M. M. 1975. Isolation, identification and synthesis of the sex pheromone of the tobacco budworm. *J. Chem. Ecol.* 1:203–214.

TUMLINSON, J. H., HEATH, R. R., and TEAL, P. E. A. 1982. Analysis of chemical communication systems of Lepidoptera, pp. 1–25, *in* B. A. Leonhardt and M. Beroza (eds.). Insect Pheromone Technology—Chemistry and Applications. American Chemical Society, Washington DC.

TURNER, J. R. G. and SHEPPARD, P. M. 1975. Absence of crossing-over in female butterflies (*Heliconius*). *Heredity* 34:265–269.

VETTER, R. S. and BAKER, T. C. 1983. Behavioral responses of male *Heliothis virescens* in a sustained flight-tunnel to combinations of seven compounds identified from female sex pheromone glands. *J. Chem. Ecol.* 9:747–759.

VICKERS, N. J. 2002. Defining a synthetic pheromone blend attractive to male *Heliothis subflexa* under wind tunnel conditions. *J. Chem. Ecol.* 28:1255–1267.

VICKERS, N. J. and BAKER, T. C. 1997. Chemical communication in heliothine moths. 7. Correlation between diminished responses to point source plumes and single filaments similarly tainted with a behavioral antagonist. *J. Comp. Physiol. A* 180:523–536.

VOS, P., HOGERS, R., BLEEKER, M., REIJANS, M., VAN DER LEE, T., HORNES, M., FRIJTERS, A., POT, J., PELEMAN, J., KUIPER, M., and ZABEA, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucl, Acids Res.* 23:4407–4414.

ZHU, J., CHASTAIN, B. B., SPOHN, B. G., and HAYNES, K. F. 1997. Assortative mating in two pheromone strains of the cabbage looper moth, *Trichoplusia ni*. *J. Insect Behav*. 10:805–817.