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Genetic basis of sex pheromone blend difference between *Helicoverpa armigera* (Hübner) and *Helicoverpa assulta* (Guenée) (Lepidoptera: Noctuidae)

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A B S T R A C T

The two closely related moth species, *Helicoverpa armigera* and *H. assulta*, are sympatric in China. Both species use a mixture of (*Z*)-11-hexadecenal (*Z*11-16:Ald) and (*Z*)-9-hexadecenal (*Z*9-16:Ald) as their sex pheromones but in widely different ratios. Hybridization and backcrossing experiments between *H. armigera* and *H. assulta* were conducted and sex pheromone compositions of the parent species, their F_1 hybrids and backcrosses were compared to study the genetic basis of the production of their sex pheromone blend composition. Results show that the difference in sex pheromone blend ratios of these *Helicoverpa* species is mainly controlled by an autosomal locus with two alleles, with the allele from *H. armigera* being almost completely dominant over that derived from *H. assulta*.

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1. Introduction

Chemical communication systems in insects have provided exciting challenges to researchers in chemistry, biochemistry, physiology, ecology, genetics, and behavior for over four decades, and much of this research has been focused on moths in the order Lepidoptera (Prestwich and Blomquist, 1987; Löfstedt, 1990, 1993; Roelofs, 1995; Cardé and Minks, 1997; Tillman et al., 1999; Millar, 2000; Roelofs and Rooney, 2003; Sheck et al., 2006). In moth species, communication during mate finding is mediated by female-produced sex pheromones usually consisting of more than one component. The species specificity of chemical components and their ratio in female sex pheromones and the specificity of the male behavioral response are often responsible for reproductive isolation between closely related species that overlap in time and space (Gemeno and Haynes, 2000). Nevertheless, although reproductive isolation operates in the field, interspecific hybridization between some closely related species of moths has been possible in the laboratory, as shown for *Apantesis* species (Bacheler and Habeck, 1974), *Heliothis* species (Laster, 1972; Teal and Oostendorp, 1995; Sheck et al., 2006), *Euxoa* species (Byers and Hinks, 1978; Teal et al., 1978; Byers et al., 1981), *Spodoptera* species (Monti et al., 1995), *Orgyia* species (Grant et al., 1975), *Ostrinia* species (Fu et al., 2005; Tabata and Ishikawa, 2005), *Ctenopseustis* species (Hansson et al., 1989), *Agrotis* species (Gadenne et al., 1997), and *Helicoverpa* species (Wang and Dong, 2001). Thus, different strains or species of moths that can hybridize have been used to dissect genetic mechanisms controlling a variety of traits related to sex communication systems such as pheromone production, detection, and behavior (Vickers, 2006). An understanding of the genetic basis and evolution of sex communication systems involved in reproductive isolation may contribute importantly to insight into the processes of species divergence and speciation (Monti et al., 1997).

The two sibling species, the cotton bollworm, *Helicoverpa armigera* and the oriental tobacco budworm, *H. assulta* (Lepidoptera: Noctuidae), are serious crop pests in China and neighboring countries (Chen, 1999). They are sympatric but have different host plant ranges. *H. armigera* is a polyphagous species feeding on more than 60 crops such as cotton, corn, tobacco, and soybean, whereas *H. assulta* is oligophagous using only some solanaceous species such as tobacco, hot pepper, and several *Physalis* species (Fitt, 1989; Chen, 1999). In spite of the significant genetic differences in their larval host plant preference (Tang et al., 2006), it is common to find mixed populations of both species on tobacco and some wild solanaceous hosts indicating the possibility that the reproductively active adults of both species encounter each other. To understand the mechanisms that are used to maintain their reproductive isolation, Ming et al. (2007) studied the pre-mating isolation between the two species and confirmed

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that the specific composition of the sex pheromone blend plays a key role.

The sex pheromone components of *H. armigera* and *H. assulta* were identified as (*Z*)-11-hexadecenal (*Z*11-16:Ald) and (*Z*)-9-hexadecenal (*Z*9-16:Ald), with *Z*11-16:Ald as the major component in *H. armigera* and *Z*9-16:Ald as the major one in *H. assulta* (Witzgall et al., 2004). In our laboratory cultures, ratios of *Z*11-16:Ald and *Z*9-16:Ald were determined as 100:2 in *H. armigera* and 6:100 in *H. assulta*, respectively (Wang et al., 2005). A biosynthetic study of sex pheromone biosynthesis showed that in these two species and their F_1 hybrids, differences in activity of the key biosynthetic enzymes, Δ9-desaturase and Δ11-desaturase, as well as reductases could result in various ratios of the *Z*9-16:Ald and *Z*11-16:Ald (Wang et al., 2005). In the present study, we conducted a series of hybridization and backcross experiments and analyzed sex pheromone blend composition in the parents, F_1 hybrids and backcross offspring to elucidate the genetic mechanism that controls the specific sex pheromone ratios in these *Helicoverpa* species.

2. Materials and methods

2.1. Insects

Larvae of *H. assulta* and *H. armigera* were collected from spatially separate tobacco and cotton fields, respectively, in a suburb of Zhengzhou, Henan province of China, and subsequently reared in a climate chamber for many generations at 27±1 °C, 55–65% relative humidity, and under a 16:8 h light–dark photoperiod cycle. Larvae were fed on artificial diet described by Wu and Gong (1997). Pupae were sexed and emerged separately. Adults were provided with a 10% honey solution.

The crossing experiments included the following crosses (female × male, GG and SS refer to *H. armigera* and *H. assulta*, respectively): GG × GG and SS × SS (parental), GG × SS and SS × GG, yielding the two reciprocal F_1 hybrids GS and SG, respectively, four backcrosses, GG × GS, SS × GS, GG × SG, and SS × SG. For each cross, 20 females and 20 males were grouped on the day of emergence in a filter paper cylinder (15 cm diameter and 15 cm height) and maintained under the conditions as described above. In this experiment, four to five replicate cages were set up. When female moths began to lay eggs, the filter paper was changed every day to collect eggs. Due to the lack of female GS offspring and low fertility of female SG in F_1 hybrids, F_2 progeny and related backcrosses could not be obtained.

2.2. Pheromone extraction and analysis

Single sex pheromone glands of 2–3 days old female adults were dissected at the 5th hour into scotophase, and extracted in 5 μl hexane at room temperature for 15 min. Chemical analysis was carried out with a Hewlett-Packard HP 5890 Series II gas chromatograph (GC) equipped with a BP-70 capillary column (50 m × 0.25 mm id, SGE, Australia) and a flame ionization detector (FID). The column temperature was programmed from 80 to 210 °C at 4°C/min, then to 240°C at 10°C/min and held for 5 min. Sex pheromone components *Z*9-16:Ald and *Z*11-16:Ald were identified by comparing their retention times with those of authentic compounds. Each female sample was characterized by a value of *r* (the ratio of *Z*9-16:Ald to *Z*11-16:Ald), and amounts of *Z*9-16:Ald and *Z*11-16:Ald were determined based on their GC peak areas (means of 16–128 individuals from each cross).

2.3. Data analysis

Variance of *r* for *H. assulta* and *H. armigera* was heterogenous and we therefore transformed *r* into another parameter *R* according to Monti et al.'s (1997) method. Of several transformations that were tested, $R = (100 \times r)^{1/6}$ was found to be appropriate. Mean and standard deviation of *R* were calculated for females of *H. assulta*, *H. armigera*, F_1 hybrids and four backcross progenies. The frequency distribution of *R* was determined and 1:1 ratio segregation of *R* in backcross progenies was tested with χ^2 analysis using SPSS 11.01 (2001).

3. Results

The distribution of *r* observed in a sample of 72 GG females showed a mean of 2.50×10^{-2} and a standard deviation of 6.64×10^{-3}, and the corresponding estimates obtained from a sample of 93 SS females were 15.00 and 5.26, respectively. Due to a scale effect, the dispersion of *r* is larger in the latter species than in the former. Thus, for statistical purposes, it was convenient to use an alternative variety *R* that would minimize the standard deviation differences between the two distributions.

The distributions of *R* in samples of parental GG and SS and their reciprocal F_1 hybrids are illustrated in Fig. 1a and b. The distribution observed in a sample of 72 GG females showed a mean (±SD) of 1.16±0.053, and the corresponding estimates obtained from a sample of 93 SS females were 3.35±0.20, respectively. The difference between the two species was distinct, and the variation among individuals within SS was larger than

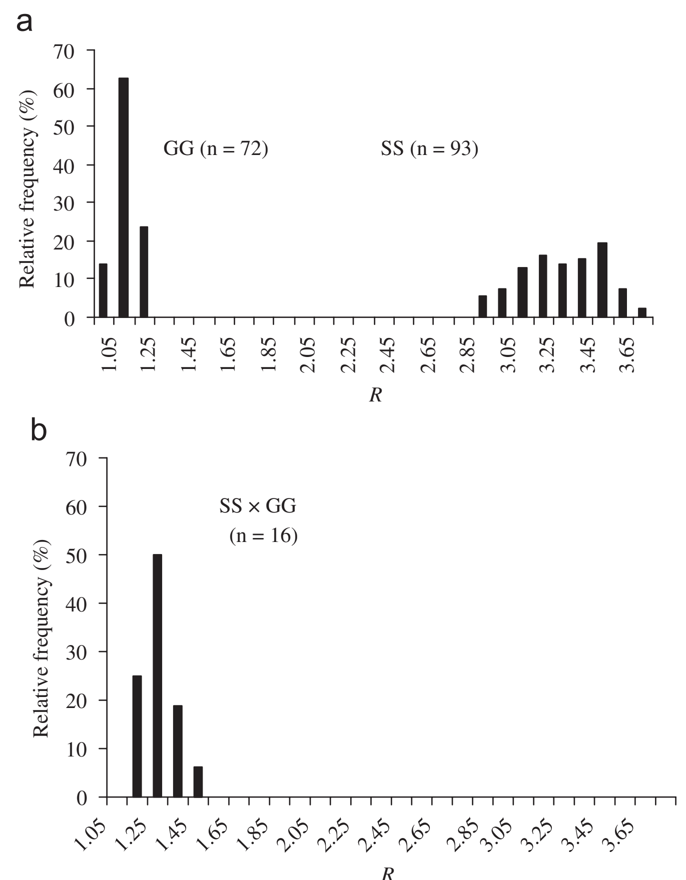

Fig. 1. Distributions of *R* in parental *H. armigera* (GG) and *H. assulta* (SS) and their F_1 hybrids (SG) (*n* = number of extracted female glands): (a) GG and SS and (b) SS × GG. $R = (100 \times r)^{1/6}$ and *r* = *Z*9-16:Ald/*Z*11-16:Ald.

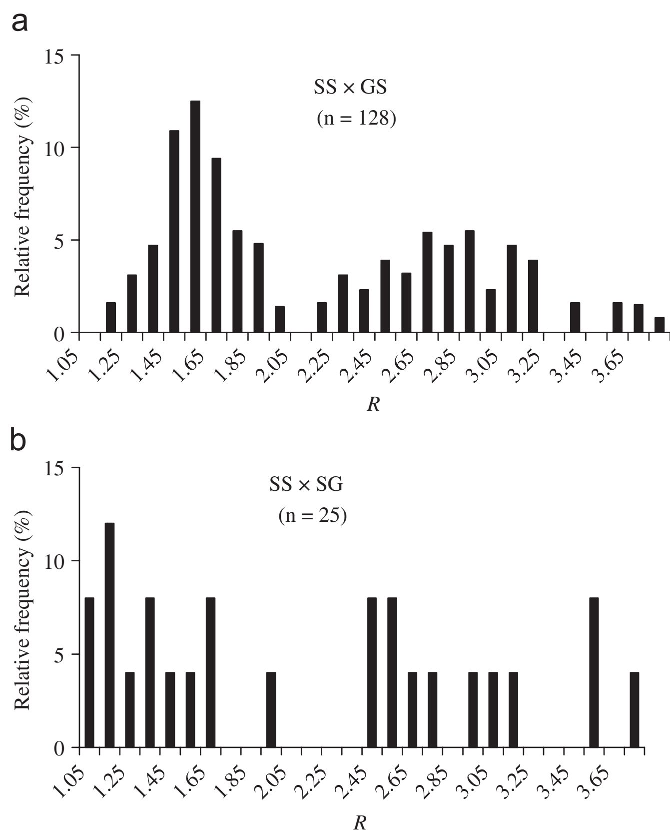

Fig. 2. Distributions of R in two backcross progenies (backcrossing to SS, n = number of extracted female glands): (a) SS × GS and (b) SS × SG. $R = (100 \times r)^{1/6}$ and r = *Z*9-16:Ald/*Z*11-16:Ald.

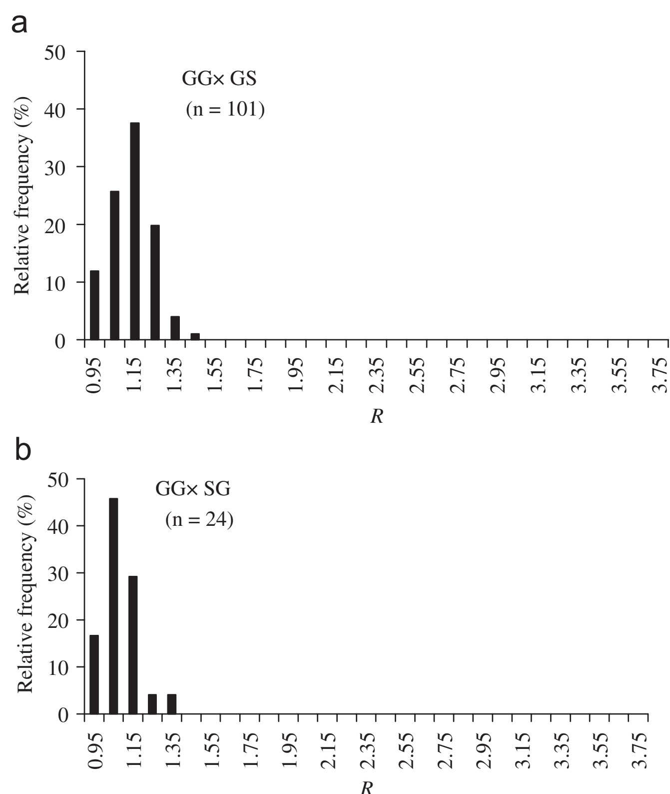

Fig. 3. Distributions of R in two backcross progenies (backcrossing to GG, n = number of extracted female glands): (a) GG × GS and (b) GG × SG. $R = (100 \times r)^{1/6}$ and r = *Z*9-16:Ald/*Z*11-16:Ald.

that within GG (Fig. 1a). The distribution observed in a sample of 16 F_1 females showed a mean ($\pm$SD) of 1.25$\pm$0.072.

Among the four backcrosses, SS × GS and SS × SG resulted in a large increase in the variation, and the genetic contributions introduced from the two original parental species segregated. The distribution of SS × GS, for which a large sample size was achieved, presented a bimodal pattern. The distribution of SS × SG presented a similar tendency although the sample size was small (Fig. 2a and b). Each population includes two groups of individuals, with R values either higher or lower than 2.05. The higher values showed means ($\pm$SD) of 2.81$\pm$0.39 and 2.95$\pm$0.48 in SS × GS and SS × SG, respectively. These values were close to that of the *H. assulta* parent. The lower values showed means ($\pm$SD) of 1.56$\pm$0.19 for SS × GS and 1.38$\pm$0.29 for SS × SG, values close to the F_1 hybrids. The observed "two-group" numbers were 69/59 for SS × GS and 13/12 for SS × SG, and these observed ratios were not significantly different from the expected ratio (for SS × GS offspring, $\chi_1^2 = 0.250$, $P > 0.01$; for SS × SG offspring, $\chi_1^2 = 0$, $P > 0.01$) and both in good agreement with the expected ratio of 1:1.

The other two backcrosses, GG × GS and GG × SG showed unimodal distributions (Fig. 3a and b). Means ($\pm$ SD) were 1.14$\pm$0.10 and 1.08$\pm$0.10 in GG × GS and GG × SG, respectively.

4. Discussion

Studies on pheromone genetics have shown different regulating mechanism among different moth species. In some species, pheromone production is controlled by a major gene. For example, in Z/E strains of the European corn borer, *Ostrinia nubilalis*, pheromone production is controlled by two alleles at a single autosomal locus (Klun and Maini, 1979), and a reductase has been implicated in the production of the specific ratios of the pheromone isomers (Zhu et al., 1996b). In the Asian corn borer, *O. furnacalis*, a mutated expression of a Δ14 desaturase gene instead of the ancestors' Δ11 desaturase gene in the European corn borer, *O. nubilalis*, probably causes the appearance of a novel pheromone component (Roelofs et al., 2002).

In the present study, pheromone gland composition analysis showed that the ratio of *Z*9-16:Ald to *Z*11-16:Ald in the F_1 hybrids (SG) was not intermediate but significantly close to that of one parental species (GG) (Fig. 1a and b), which suggests almost complete dominance of the GG phenotype for the pheromone blend ratios. Therefore, among all the female offspring derived from the backcrosses to the *H. armigera* parent (GG), it was impossible to classify them as being either heterozygous (GS) or homozygous (GG), whereas among all the female offspring derived from the backcrosses to the *H. assulta* parent (SS), they could be easily classified as being either heterozygous (GS) or homozygous (SS).

According to our results, the genetic contributions derived from the two parental species segregated, and the backcross SS × GS and SS × SG yielded a bimodal distribution in a 1:1 ratio. Results from the other two backcrosses, GG × GS and GG × SG are consistent with the hypothesis that each progeny includes 50% of individuals closely similar to F_1 hybrid females and 50% closely similar to *H. armigera* females. Each backcross distribution is composed of two undivided parts. These results thus point to a major dominant factor controlling the inheritance of the pheromone blend ratios.

Sex determination in Lepidoptera is of the ZZ/ZW type with males being the homogametic sex, and the potential inheritance scheme that could explain the observed blend ratios is either autosomal or through Z chromosome inheritance. Due to a single dominant genetic factor resulting in the differences of sex

Table 1
Observed and expected inheritance patterns of female sex pheromone ratio in the hybrids of *H. armigera* (GG) and *H. assulta* (SS) based on autosomal or Z-linked inheritance

Cross type	Parentage	Inferred female genotype	Phenotype of progenies if *R*-dominance	
	Female × male		Expected[a]	Observed
F_1a	GG × SS Z_GW_G × ZsZs	GS ZsW_G	G S	[b]
F_1b	SS × GG ZsWs × Z_GZ_G	SG Z_GWs	G G	G
BcaG	GG × GS Z_GW_G × Z_GZs	1/2GG, 1/2GS 1/2Z_GW_G, 1/2ZsW_G	G 1/2G, 1/2S	G
BcaS	SS × GS ZsWs × Z_GZs	1/2SG, 1/2SS 1/2Z_GWs, 1/2ZsWs	1/2G, 1/2S 1/2G, 1/2S	1/2G, 1/2S
BCbG	GG × SG Z_GW_G × ZsZ_G	1/2GS, 1/2GG 1/2ZsW_G, 1/2Z_GW_G	G 1/2S, 1/2G	G
BCbS	SS × SG ZsWs × ZsZ_G	1/2SS, 1/2SG 1/2ZsWs, 1/2Z_GWs	1/2S, 1/2G 1/2S, 1/2G	1/2G, 1/2S

[a] The phenotype of progenies as expected based on the single-major-gene model for pheromone ratio.
[b] Females were not obtained in F_1 progenies.

pheromone ratio, autosomal and Z-linked inheritance should produce diagnostic differences in phenotype frequencies in some of the crosses. If sex pheromone blend ratio distributions of F_1 hybrids from reciprocal crosses were very similar and the average ratios were the same in either reciprocal cross, it would indicate an autosomal pattern of inheritance, otherwise, it would be a Z-chromosome pattern of inheritance. Because there were no females in F_1 hybrids derived from female *H. armigera* (GG) crossed with male *H. assulta* (SS), it is impossible to compare the female pheromone blend ratios of the two reciprocal crosses. However, two (SS × GS and SS × SG) of the four backcrosses would produces equal phenotype frequencies under sex-linked and autosomal inheritance, whereas the other two (GG × GS and GG × SG) would segregate differentially under the two schemes (Table 1). The observed phenotypic patterns of backcross females derived from either reciprocal F_1 hybrid males mated with *H. armigera* (GG) females may be used for a preliminary genetic analysis, and are as a matter of fact in reasonably good agreement with the expected ratio under autosomal inheritance (Table 1), which suggests that the major genetic factor controlling the pheromone blend ratios is carried by an autosome rather than by the Z chromosome.

On the other hand, the range of variation in pheromone ratios was narrow in the parental females (Fig. 1a), but relatively wide in the backcross females (Fig. 2a and b). These results implied that in addition to the single major autosomal gene, some modifier genes could also be involved in the regulation of pheromone ratio, as was verified in *Ostrinia* species (Zhu et al., 1996a).

According to prior pheromone biosynthesis studies, the production of aldehyde pheromone compounds generally starts from the desaturation of the *de novo* synthesized saturated fatty acid, coupled with carbon chain shortening or elongation to form the fatty acid precursors with specific length and double bond location. These precursors subsequently undergo the selective reduction and terminal oxidization (Bjostad et al., 1987; Teal and Tumlinson, 1988; Jurenka, 2003). In a previous study, we proved that the terminal oxidase does not have substrate specificity in any of the parental species (Wang, H.L., Zhao, C.H., Yan, Y.H., Wang, C.Z., unpublished data), and the ratio of the two fatty acid biosynthetic precursors, *Z*9-16:acid and *Z*11-16:acid, was similar to that of the corresponding aldehydes, *Z*9-16:Ald and *Z*11-16:Ald, in *H. assulta*, but significantly different in *H. armigera* and F_1 hybrid (Wang et al., 2005). These results suggested that the reductase does not show significant specificity to the fatty acid precursors in *H. assulta*, and therefore the desaturation process should play a major part in the pheromone ratio regulation, whereas in *H. armigera* and F_1 hybrid, the reduction step could be more specific, and as a result their specific pheromone ratio could be regulated by the interaction of the desaturase and reductase.

Recently we evaluated genetic differentiation of *H. armigera* and *H. assulta*, and determined the numbers of species-specific AFLP markers (Ming and Wang, 2006), according to the method that has been applied on *Heliothis* species (Groot et al., 2004; Sheck et al., 2006). Further studies such as AFLP marker-based mapping of backcross families with *H. assulta* direction and fine-scale mapping and cloning of quantitative trait loci (QTL) should be helpful to determine which autosome in *Helicoverpa* species carries the QTL and the related gene that controls pheromone blend ratio.

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