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## IDENTIFICATION OF FALL ARMYWORM (LEPIDOPTERA: NOCTUIDAE) HOST STRAINS BASED ON MALE-DERIVED SPERMATOPHORES

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## ABSTRACT

Laboratory experiments were designed to identify the host strain paternity of fall armyworm *Spodoptera frugiperda* (J. E. Smith) mated females. In no-choice tests, corn or rice strain females were placed in cages with males of the opposite strain. After 48 h, females were dissected and spermatophores were removed. Molecular markers in the *cytochrome oxidase I (COI)* gene were used to identify host strain identity from the spermatophores and results showed the host strain pattern of the mating males. In choice tests, females of either strain were placed in cages with males of both strains. After 48 or 96 h, spermatophores were dissected and were molecularly analyzed to identify the host strain of the mating males. Corn and rice strain females contained spermatophores from males of both strains, indicating that interstrain mating commonly occurs in the laboratory. The analysis of the spermatophores isolated from mated females provides a convenient means of identifying the strain of the mated male. This technique has the promise of being able to directly measure interstrain mating in wild populations.

Key Words: *Spodoptera frugiperda*, host strains, spermatophores, mating

## RESUMEN

Experimentos de laboratorio fueron diseñados para identificar la paternidad de hembras apareadas de diferente sepa de cogollero, *Spodoptera frugiperda* (J. E. Smith) según el hospedero. En pruebas de “no opción”, hembras de sepas de maíz y arroz fueron puestas en jaulas con machos de la sepa opuesta. Después de 48 horas, las hembras fueron disectadas y los espermatóforos fueron quitados. Se utilizaron marcadores moleculares en el gene citocromo oxidase (*COI*) tomados de los espermatóforos para identificar la sepa de cogollero y los resultados mostraron el patrón de la sepa de los machos apareados. En pruebas de “con opción”, escoger, hembras de cualquiera de las dos sepas fueron puestas en jaulas con machos de ambas sepas. Después de 48 o 96 horas, los espermatóforos fueron disectados y analizados molecularmente para identificar la sepa de los machos apareados. Hembras de la sepa de maíz y arroz tenían espermatóforos de ambas sepas. Este indica que el apareamiento entre las dos sepas es común en el laboratorio. El análisis de los espermatóforos aislados de hembras apareadas provee una manera conveniente para identificar la sepa del macho apareado. Esta tecnica tiene la promesa de poder medir directamente el apareamiento entre las sepas en poblaciones salvajes.

Fall armyworm *Spodoptera frugiperda* (J. E. Smith) provides a potentially useful system to study sympatric speciation. This migratory, polyphagous noctuid moth attacks a wide variety of crops throughout the Nearctic and Neotropical Western Hemisphere (Luginbill 1928; Sparks 1979). The species is composed of 2 morphologically identical strains that are defined by their host plant preferences (Nagoshi & Meagher 2004a; Nagoshi & Meagher 2008). One strain was identified from populations feeding on corn and sorghum (corn strain) and the other was identified from populations feeding on rice (*Oryza sativa* L.) and forage grasses (*Cynodon* spp.) (rice strain) (Pashley et al. 1985; Pashley 1986; Pashley et al. 1987). The 2 strains can be distinguished by genetic markers (Levy et al. 2002; Nagoshi & Meagher 2003; Nagoshi et al. 2006b). Pheromone trapping studies in agricultural habitats routinely attract males of both strains, though the

proportion will vary depending on the dominant plant type, indicating that the strains overlap substantially in their distribution and are mutually attracted to a common pheromone source (Meagher & Nagoshi 2004; Nagoshi & Meagher 2004b).

The maintenance of strain integrity in sympatric populations suggests some restrictions in interstrain hybridization (Prowell et al. 2004; Nagoshi et al. 2006a). Laboratory experiments presented evidence of directional premating barriers where matings between corn strain females and rice strain males did not result in the transfer of spermatophores, but the opposite cross was successful (Pashley & Martin 1987). They also found that hybrid females produced by successful interstrain crosses could only successfully mate with their hybrid brothers. In comparison, hybrid males could mate with females of either strain with near normal fertility. The strongest premat-

ing restriction to hybrid formation so far observed is the temporal partitioning of mating behavior, as laboratory experiments showed that corn strain females initiated calling and copulated earlier in the scotophase than rice strain females (Pashley et al. 1992). Similar results were obtained in a more recent set of experiments with different laboratory corn and rice strain colonies (Schöft et al. 2009). In these studies it was shown that the timing of female calling was determined by maternal effects, while copulation was influenced by a combination of maternal and dominant autosomal factors.

However, these strain-specific mating biases were not observed by other groups working with different colonies (Whitford et al. 1988; Quisenberry 1991). The 2 strains successfully hybridized in both directions with no difference in fecundity and the hybrids themselves showed no mating specificity. We obtained similar results with colonies in which the strains were defined by molecular markers (Nagoshi & Meagher 2003; unpublished results). This inability to observe strain-specific mating could be an artifact of prolonged artificial rearing, as suggested by Pashley (1993), and suggests that the behaviors are relatively labile and readily influenced by environmental factors or genetic inbreeding.

The capacity for interstrain hybridization is of importance to understanding how the integrity of these sympatric strains is maintained and for controlling the infestations of this important agricultural pest. With respect to the latter, there are several observations that the viability and development of the 2 strains differs between plant hosts (Pashley 1986, 1988; Whitford et al. 1992; Pashley 1993; Pashley et al. 1995; Meagher et al. 2004). The 2 strains also differ in their sensitivity to a variety of pesticides (McCord & Yu 1987; Yu 1991; Veenstra et al. 1995; Adamczyk et al. 1997; Yu 1999), and possibly to Bt susceptibility as well, although in many cases the strain identity of the lines being tested was unclear (Adamczyk et al. 1997; Lynch et al. 1999; Adamczyk et al. 2001; Dequech et al. 2005; Polanczyk & Alves 2005; Monnerat et al. 2006; Williams et al. 2006; Chilcutt et al. 2007).

A major problem in addressing this issue is the difficulty in performing such studies, particularly in the field. Current methods require continued observation during the scotophase period and the collection of the mated pairs to determine the molecular markers carried by each parent. In this paper we describe a method for identifying the occurrence of successful interstrain matings that does not require monitoring, collecting, or analysis of the male parent. Instead, spermatophores, which are male products transferred to the female during a successful copulation (LaMunyon 2000; Blanco et al. 2006), were dissected from the mated female and used to identify the strain of

the male. We used this method in no-choice and choice experiments to examine the interstrain mating capacity of several corn strain and rice strain colonies, including those shown by Schöft et al. (2009) to display strain-specific temporal differences in mating behavior, as a proof-of-concept to developing a direct method of measuring the frequency of interstrain mating in the field.

## MATERIALS AND METHODS

### Generation of Strain-Specific Cultures

To generate the cultures used in these studies, larvae were collected from either corn (corn strain) or pasture grass (rice strain). It was previously reported that about 20% of the larvae typically collected from corn is of the rice strain, a proportion that could even be higher depending on the time of year (Nagoshi & Meagher 2004b; Nagoshi et al. 2007). To insure the strain-specificity of each culture, the larvae from each plant host were raised to adulthood and pair-mated. After oviposition, the parents were analyzed for strain-identity by the COI markers (Nagoshi & Meagher 2003). Only progeny from parents where both were of the appropriate strain were used to generate the laboratory cultures.

Culture procedures followed Stuhl et al. (2008). Adults were placed in cylindrical screen cages (28 cm height, 21 cm diameter) and supplied with a 2% sugar-honey solution for nourishment. Paper towels (Sparkle™, Georgia-Pacific, Atlanta, GA) were stretched at the tops of the cages as an oviposition substrate. Emerging neonates were placed in rearing tubs (Rubbermaid No. 4025, 9.1 l, Fairlawn, OH) that had plastic grids (29 × 17.5 cm) on the bottom. Larvae were raised on a pinto bean artificial diet according to the procedures of Guy et al. (1985). After about 23 d, pupae were removed from the tubs, sexed, and adults that emerged were placed in screen cages. Larvae and adults were reared in incubators or large rearing units at ≈3°C, 70% RH, and 14L:10D photoperiod.

A total of 5 colonies were produced by this method, 3 corn strain: 'CS-JS3' from larvae collected in sweet corn in Miami-Dade Co., FL (13 months in culture), and 2 cultures from field corn in Alachua Co., FL ('CS-Hague', 10 months and 'CS-DRU', 6 months), and 2 rice strain: 'RS-Ona' from larvae collected in Hardee Co., FL in pasture grasses (22 months) and 'RS-MS' from larvae collected in Washington Co., MS in pasture grasses (18 months).

### No Choice and Choice Tests

Each of the 5 colonies was tested separately. Two experiments (no-choice and choice) were conducted and adults used were between 2 and 5 d

old. In the no-choice experiment, 10 females and males each of opposite strains (5 RS-MS females × 5 CS-Hague males + 5 RS-Ona females × 5 CS-DRU males in separate cages; 10 CS-DRU females × 10 RS-Ona males) were placed in a screen cage (24 × 24 × 24 cm) with 2% honey/sugar solution for nourishment. After 96 h, females were frozen (-20°C) and later dissected to remove the spermatophore(s). In the choice experiment, 6 females of 1 strain were placed in the screen cage with 4 rice strain and 4 corn strain males. Twelve trials were completed with corn strain females (CS-JS, CS-Hague, or CS-DRU) while 10 trials were completed with rice strain females (RS-MS or RS-Ona). Females were held for either 48 or 96 h before being frozen and dissected. Spermatophores located in the female's corpus bursae were gently removed in 70% ethanol. Care was taken to keep the three sections (bulbous corpus, stem-like collum, and hook-like frenum) in one piece and to remove as much of the tissue around the spermatophore as possible to avoid contamination from the female. Each spermatophore was placed in 70% ethanol and in a separate snap vial before being placed in a freezer.

#### Spermatophore DNA Preparation

Individual spermatophores were homogenized in 800 µL of Genome lysis buffer (Zymo Research, Orange, CA) in a dounce homogenizer. The homogenate was transferred to a 1.5-mL microcentrifuge tube and incubated at 55°C for 5 min. The supernatant was transferred to a Zymo-Spin I column (Zymo Research, Orange, CA) and processed according to manufacturer's instructions. The DNA preparation was collected in a final volume of 20 µL with distilled water, sufficient for 2 PCR amplification reactions.

#### Spermatophore PCR Analysis For Strain Identity

Strain-identity in spermatophores was determined by PCR amplification (PTC-200 Thermo Cycler, MJ Research, Watertown, MA) by 3 methods defined by different primer pairs. PCR amplification was performed in a 30-µL reaction mix containing 3 µL 10X manufacturer's reaction buffer, 1 µL 10mM dNTP, 0.5 µL 20 µM primer mix, 10 µL DNA template (between 0.05-0.5 µg), and 0.5 unit Taq DNA polymerase (New England Biolabs, Beverly, MA). The thermocycling program was 94°C (1 min), followed by 33 cycles of 92°C (30 s), 56°C (45 s), 72°C (45 s), and a final segment of 72°C for 3 min. Digestions with restriction enzymes (New England Biolabs, Beverly, MA) used manufacturer-provided buffers. Each reaction used 10-20 units of restriction enzyme and was incubated at 37°C for 3 h to overnight. Negative controls were performed with the same reaction mixture but with no DNA template. For

gel electrophoresis, 6 µL of 6X gel loading buffer was added to each reaction and the entire sample run on 2% agarose horizontal gel containing Gel-Red (Biotium, Hayward, CA) in 0.5X Tris-borate buffer (TBE, 45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0). Fragments were visualized with a long-wave UV light box.

Primers were synthesized by Integrated DNA Technologies (Coralville, IA). Amplification of the *COI* region by Method I used the primer pair *COI-58F* (5'- GGAATTTGAGCAGGAATAGTAGG-3') and *COI-1059R* (5'- ACACCTGTTAATCCTCCTACAG-3') to produce a 1-kb fragment (Nagoshi et al. 2006b). Method II used primers *COI-893F* (5'-CACGAGCATATTTTACATCWGCA-3') and *COI-1472R* (5'- GCTGGTGTAAATTTTGATATC-3') to produce a 571-bp fragment. Method III used primers *COI-259F* (5'- CCTGATATAGCTTTCACAG - 3') and *COI-857R* (5'- GCAGATGTAAAATATGCTCGTG - 3') to produce a 654-bp fragment. Restriction digest was the same as Method I.

#### Adult Moth Strain Analysis

Individual specimens were homogenized in 4 mL of phosphate buffered saline (PBS, 20 mM sodium phosphate, 150 mM NaCl, pH 8.0) in a 15 mL-test tube with a tissue homogenizer (PRO Scientific Inc., Oxford, CT). Cells and tissue were pelleted by centrifugation at 6000g for 5 min at room temperature. The pellet was resuspended in 800 µL cell lysis buffer (0.2 M sucrose, 0.1 M Tris-HCl at pH 8.0, 0.05 M EDTA, and 0.5% sodium dodecyl sulfate), transferred to a 1.5- or 2.0-mL microcentrifuge tube and incubated at 55°C for 5 min. Proteins were precipitated by the addition of 100 µL of 8M potassium acetate. The supernatant was transferred to a Zymo-Spin III column (Zymo Research, Orange, CA) and processed according to manufacturer's instructions. The DNA preparation was increased to a final volume of 40 µL with distilled water. PCR amplification and restriction digest of the mitochondrial *COI* gene was as described for spermatophore Method I except that 1 µL of the DNA template (between 0.05-0.5 µg) was used for the amplification reaction.

## RESULTS AND DISCUSSION

#### Determining Spermatophore Strain Identity

We wanted to test the feasibility of using spermatophores dissected from mated fall armyworm females to identify the strain of the male involved. Noctuid spermatophores range in size from 0.6-0.8 mg (He & Tsubaki 1992), so we used PCR amplification methods that can generate microgram amounts of targeted DNA sequences from very low levels of starting material. Two

methods were initially used to identify strain-identity in spermatophores. In the preferred method (designated I), a 1-kb PCR amplified product was generated carrying both a single rice strain and corn strain specific *Msp*I site (Fig. 1; Nagoshi et al. 2008). Digestion by *Msp*I will therefore produce strain diagnostic bands that can be distinguished from any uncut fragment resulting from a failure of restriction enzyme digestion.

If this procedure did not provide an unambiguous strain identity, a second method (designated II) was attempted to at least identify spermatophores of the rice strain. Method II generates a 571-bp product that because of its smaller size should be more efficiently amplified. The fragment contains a strain-specific polymorphism that disrupts a single *Eco*RV site in the corn

strain but not the rice strain (polymorphism 1182 in Nagoshi et al. 2010), producing diagnostic bands upon digestion with this enzyme. However, this method is ambiguous for the corn strain because the diagnostic pattern cannot be distinguished from incomplete restriction digestion. In subsequent analyses, a modification of method I was used in which an internal pair of primers amplified a fragment about the size of that produced by method II, but producing diagnostic PCR bands for both strains after *Msp*I digestion (Fig. 1).

#### Spermatophores Identify Interstrain Mating

Representatives from each laboratory culture were tested for interstrain mating capability under laboratory conditions. Reciprocal no-

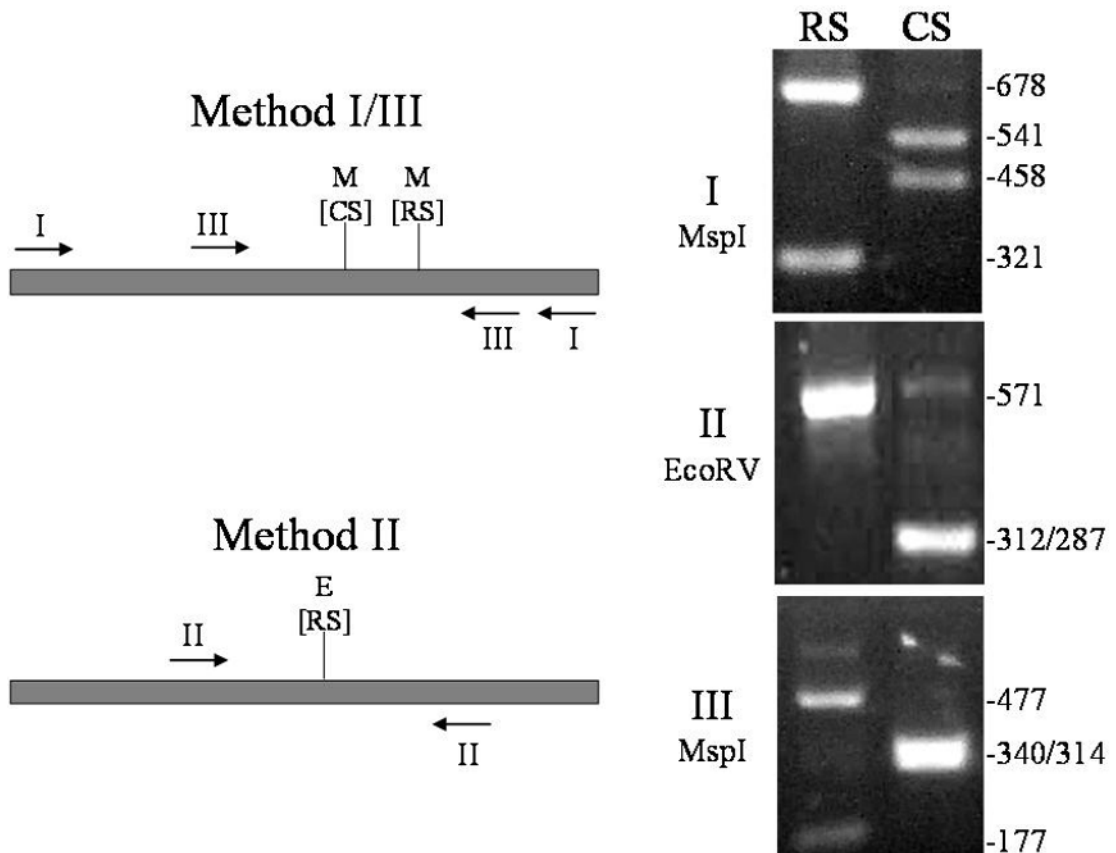


Fig. 1. Molecular markers in the COI gene that establish host strain identity used for the analysis of spermatophores (corn strain = CS, rice strain = RS). Shown are agarose gels with PCR amplified bands cut with the designated restriction enzyme. Methods I and III amplify overlapping regions with the method III primer pair internal to that of method I. Strain-specific *Msp*I sites are present on the fragment that produces diagnostic patterns for strain identity. Method II amplifies a different portion of the COI gene that contains a corn strain specific *Eco*RV site. A 571-bp fragment is frequently observed even when the primary bands are those associated with the rice strain. This fragment is either due to incomplete restriction digest or contamination with corn strain tissue. Numbers on the side of the gel picture are sizes in base pairs (CS = corn strain; RS = rice strain).

choice experiments were performed and successful mating was defined as the presence of spermatophores in the females. After 96 h, 7 corn strain and 10 rice strain females successfully mated with males of the opposite strain to produce 11 ( $1.57 \pm 0.3$  spermatophores per mated female) and 13 ( $1.3 \pm 0.15$  spermatophores per mated female) spermatophores, respectively. Multiple spermatophores were found in 3 of the corn strain females and 3 of the rice strain females, indicating that females mating to multiple partners were common. The *Msp*I enzyme digestion reactions were successful for 10 spermatophores contained in corn strain females and 8 contained in rice strain females. Of the 10 spermatophores from corn strain females all were identified as rice strain by molecular analysis (Table 1). Similarly, in the reciprocal cross, all 8 spermatophores from rice strain females tested showed the *COI* markers consistent with the corn strain.

In the choice experiments, females were simultaneously presented males of both strains. A high percentage of both strain females mated successfully (corn strain  $81.8 \pm 3.8\%$ ; rice strain  $88.2 \pm 3.6\%$ ) and contained more than 2 spermatophores per mated female (corn strain  $2.04 \pm 0.14$ ; rice strain  $2.22 \pm 0.18$ ). For the DNA analysis, 15 spermatophores were isolated from 10 corn strain females that were successfully mated. Seven spermatophores exclusively displayed the same corn strain markers as the mother, whereas the remaining 8 spermatophores expressed the rice strain marker indicating interstrain mating (Table 1). Of the 20 spermatophores analyzed from rice strain females, 10 were of rice strain males and 10 were indicative of interstrain mating. These observations demonstrate that there are no discernible barriers to mating between the 2 strains under our laboratory conditions.

Our inability to replicate the strain mating biases described by Pashley & Martin (1987) sug-

gests that this behavior is easily lost after prolonged laboratory culturing or is highly variable within the fall armyworm population. There is evidence that substantial interstrain hybridization occurs in the wild. Multi-locus analysis of strain-specific genetic markers can be used as an indirect measure of interstrain hybrids, producing estimates of hybridization frequency ranging from 16-26% (Prowell et al. 2004; Nagoshi et al. 2006a). The data suggest that matings of rice strain females to corn strain males were more frequent than the reciprocal, the same bias observed by Pashley & Martin (1987) in laboratory studies (Nagoshi & Meagher 2003; Nagoshi et al. 2006a; Nagoshi et al. 2008). If these estimates of hybridization in the wild are correct, then hybrids make up a large part of the sampled population with potentially different behaviors and physiologies than the parental strains. This would indicate a more genetically complex situation for fall armyworm that would complicate efforts to control and predict the infestations of this important agricultural pest.

To accurately evaluate the importance of hybrid formation in fall armyworm field populations, we developed a methodology that can directly measure the frequency of hybrid formation in different habitats and seasons. Spermatophores have previously been used to clarify host races in another sympatric species by measuring for differences in stable isotope frequencies (Maulausa et al. 2005). Our research is the first to identify the host strain paternity of mated females with molecular markers. This makes it possible to detect interstrain matings solely from the collection of females, thereby facilitating the direct determination of hybrid frequency in different habitats and seasons. As proof-of-concept, we show in preliminary experiments that the strains represented by our colonies are capable of substantial interstrain mating and show no indication of strain preference under laboratory conditions.

TABLE 1. RESULTS OF NO-CHOICE AND CHOICE MATING EXPERIMENTS BASED ON SPERMATOPHORES AS A MEASURE OF SUCCESSFUL MATING. CORN STRAIN = CS; RICE STRAIN = RS.

Strain of ♀ parent	Strain of ♂ parent(s)	# ♀ parents dissected	♀s with >1 spermatophore	Spermatophore COI haplotype	
				Same strain as ♀ parent	Opposite strain of ♀ parent
CS	RS	7	3	0	10 (10) <sup>b</sup>
RS	CS	10	4	0	8
CS	CS and RS	10	5 [2] <sup>a</sup>	7	8 (2) <sup>b</sup>
RS	CS and RS	19	11 [5] <sup>a</sup>	10	10

<sup>a</sup>brackets indicate number of clusters where spermatophores had different strain identity.  
<sup>b</sup>parentheses indicate number analyzed by Method II.

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