New Restriction Fragment Length Polymorphisms in the Cytochrome Oxidase I Gene Facilitate Host Strain Identification of Fall Armyworm (Lepidoptera: Noctuidae) Populations in the Southeastern United States

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ABSTRACT Several restriction sites in the cytochrome oxidase I gene of fall armyworm, *Spodoptera frugiperda* (J.E. Smith), were identified by sequence analysis as potentially being specific to one of the two host strains. Strain specificity was demonstrated for populations in Florida, Texas, Mississippi, Georgia, and North Carolina, with an AciI and SacI site specific to the rice (*Oryza* spp.)-strain and a BsmI and HinfI site joining an already characterized MspI site as diagnostic of the corn (*Zea mays* L.)-strain. All four of these sites can be detected by digestion of a single 568-bp polymerase chain reaction-amplified fragment, but the use of two enzymes in separate digests was found to provide accurate and rapid determination of strain identity. The effectiveness of this method was demonstrated by the analysis of almost 200 adult and larval specimens from the Mississippi delta region. The results indicated that the corn-strain is likely to be the primary strain infesting cotton (*Gossypium* spp.) and that an unexpected outbreak of fall armyworm on the ornamental tree *Paulownia tomentosa* (Thunb.) Sieb. & Zucc. ex Steud. was due almost entirely to the rice-strain.

KEY WORDS *Spodoptera frugiperda*, cotton, restriction fragment length polymorphism

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a significant and periodic pest of several crops, including corn, *Zea mays* L., and forage and turf grasses (Luginbill 1928, Sparks 1979). The species overwinters in southern Florida and southern Texas and in mild winters along the Gulf Coast. These areas serve as the source of migrant populations in the eastern and central United States and Canada (Barfield et al. 1980, Mitchell et al. 1991). Analysis of electrophoretic protein variants found a strong correlation between plant host and the presence of particular allozymes, identifying one strain (designated corn-strain or C-strain) primarily associated with large grasses, such as corn and sorghum, *Sorghum bicolor* (L.) Moench, and another strain, rice (*Oryza* spp.)-strain (R-strain) that fed on smaller grasses, including Bermuda grass, *Cynodon dactylon* (L.) Pers. (Pashley 1986, Pashley et al. 1987b, Pashley 1988b). Additional behavioral and physiological distinctions between strains have been reported, including differences in pesticide resistance, susceptibility to different plant cultivars, mating partner choice, and development on different food sources (Pashley and Martin 1987; Pashley et al. 1987a, 1992, 1995; Pashley 1988a; Jamjanya et al. 1990; Veenstra et al. 1995; Adamczyk et al. 1997).

Strain-specific morphological characters have yet to be found, so strain identification remains dependent on molecular markers. Several studies have identified restriction site differences between strains, and the best characterized of these differences are in mitochondrial genes (Pashley 1986, Pashley 1989, Lu et al. 1992, Pashley and Ke 1992, Lu and Adang 1996). In particular, an MspI restriction enzyme polymorphism was identified in the mitochondrial *cytochrome oxidase I* (*COI*) gene that is diagnostic of strain identity and for which a polymerase chain reaction (PCR)-based method of detection is available that allows the analysis of single specimens (Lu and Adang 1996, Levy et al. 2002, Meagher and Gallo-Meagher 2003). We have previously used the presence of this restriction fragment length polymorphism (RFLP) as the primary indicator of C-strain identity, noted as mt^C, with its absence denoting the R-strain, or mt^R (Nagoshi and Meagher 2003, Meagher and Nagoshi 2004, Nagoshi

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Table 1. Source locality and host information for specimens tested for host strain identity

n	Location	Collection	Plant/habitat	Date	Collector
80	Belle Glade, FL	Larvae	Corn	Sept.–Nov. 2003	G. Nuessly
47	Avon Park, FL	Pheromone	Cornfield	Aug.–Oct. 2003	G. Nuessly
35	Ona, FL	Pheromone	Pasture	April 2004	R. Meagher
171	Miami-Dade Co., FL	Pheromone	Cornfield/pasture	2003–2004	R. Meagher
24	Miami-Dade Co., FL	Larvae	Corn	Jan. 2004	R. Meagher
48	Collier Co., FL	Pheromone	Pasture	April–Dec. 2003	R. Meagher
31	Griffin, GA	Pheromone	Pasture	Aug.–Sept. 2004	K. Braman
20	Weslaco, TX	Pheromone	Sorghum field	April 2004	W. Warfield
21	Various locations, NC	Pheromone	Pasture	Aug.–Sept. 2004	R. Brandenburg
168	Washington Co., MS	Pheromone	Cotton field	June–Nov. 2004	J. Adamczyk
48	Washington Co., MS	Larvae	*P. tomentosa*	Oct.–Nov. 2004	J. Adamczyk
20	Washington Co., MS[a]	Larvae	Cotton laboratory colony	Sept. 2003	J. Adamczyk

[a] Location where the original colony founders were collected.

and Meagher 2004). A second genetic marker is a polymorphic HinfI site in the mitochondrial *ND1* gene (Pashley 1989, Prowell et al. 2004). This site is present predominantly in fall armyworm carrying enzymatic and allozyme markers characteristic of the R-strain. Although it is likely that the above-described polymorphisms in the *ND1* and *COI* genes identify the same strain-specific mitochondrial lineages, this has not been directly demonstrated.

Understanding the population dynamics of the two strains will be important to both predicting and controlling future infestations. In our experience, PCR-RFLP is the only practical means of individually analyzing large numbers of field-collected fall armyworm specimens for strain identity, particularly when the quality of the specimens is compromised by prolonged exposure to environmental conditions. However, reliance on a single restriction site polymorphism, such as in the *COI* (MspI) or *ND1* (HinfI) genes, can give rise to inaccuracies from a failed or incomplete restriction digest. The use of both markers obviates this problem, but requires a second PCR amplification either as a separate or multiplex reaction, increasing the expense and complication of the procedure. A simpler methodology would be if a single PCR-amplified fragment could be tested for two or more restriction polymorphisms, with at least one polymorphism specific for each strain.

In this article, we identify and characterize RFLPs in the COI gene that allow this strategy of strain determination. The utility of this approach was demonstrated by the examination of several hundred specimens from Mississippi, Georgia, North Carolina, Texas, and Florida. The method was successfully applied to adults captured in pheromone traps and stored frozen as well as ethanol-preserved larvae, and the data obtained provided evidence supporting the contention that the C-strain is the primary population infesting cotton (*Gossypium* spp.) in the Mississippi delta region.

Materials and Methods

Trap Collection and Sites. Fall armyworm specimens were obtained at several locations in the southern United States by using pheromone trapping and larval collections (Table 1). Adult males were collected using pheromone traps as described previously (Meagher and Nagoshi 2004). Standard plastic Unitraps were baited with a commercially available fall armyworm pheromone (Scenturion Inc., Clinton, WA) and contained insecticide strips (Hercon Environmental Co., Emigsville, PA). Field-collected adult moths were collected from traps after 1–14-d sample periods, depending on the population level and location, during which time they are exposed to field conditions. After collection, specimens were stored at −20°C. Larvae were collected from host plants and identified by morphological criteria. They were then reared on artificial media in the laboratory (Guy et al. 1985). DNA was isolated from these specimens either as adults or late postfourth instars. Egg masses were collected from the ornamental tree royal paulownia, *Paulownia tomentosa* (Thunb.) Sieb. & Zucc. ex Steud. (Scrophulariaceae), raised until postfourth instar on artificial media, and then preserved in 100% ethanol until DNA isolation.

DNA Sequence Analysis. All DNA sequences were obtained from National Center for Biotechnology Center GenBank. The sequence from the R-strain COI gene (accession no. U72977) was compared with that from the C-strain (accession no. U72974), and base pair differences associated with altered restriction sites were identified. DNA comparisons and restriction site mapping were performed using the DS Gene program (Accelrys, San Diego, CA).

DNA Preparation. Individual specimens were homogenized in 1 ml of homogenization buffer (0.03 M Tris-HCl, pH 8.0, 0.1 M NaCl, 0.2 M sucrose, 0.01 M EDTA, pH 8.0, and 0.5% Triton X-100) in a 5-ml Dounce homogenizer by using either a hand pestle or a motorized mixer. To remove large debris, the homogenate was filtered through a 1-ml plastic syringe plugged with cheesecloth (prewet with distilled water) into a 2-ml microfuge tube. Cells and nuclei were pelleted by centrifugation at 12,000 × *g* for 5 min at 4°C. The pellet was resuspended in 600 μl of nuclei buffer (0.01 M Tris-HCl, pH 8.0, 0.35 M NaCl, 0.1 M EDTA, and 1% *N*-lauryl sarcosine), and extracted with 400 μl of phenol/chloroform (1:1). The supernatant was transferred to a DNA Clean and Concentrator-5 column (Zymo Research, Orange, CA) according to

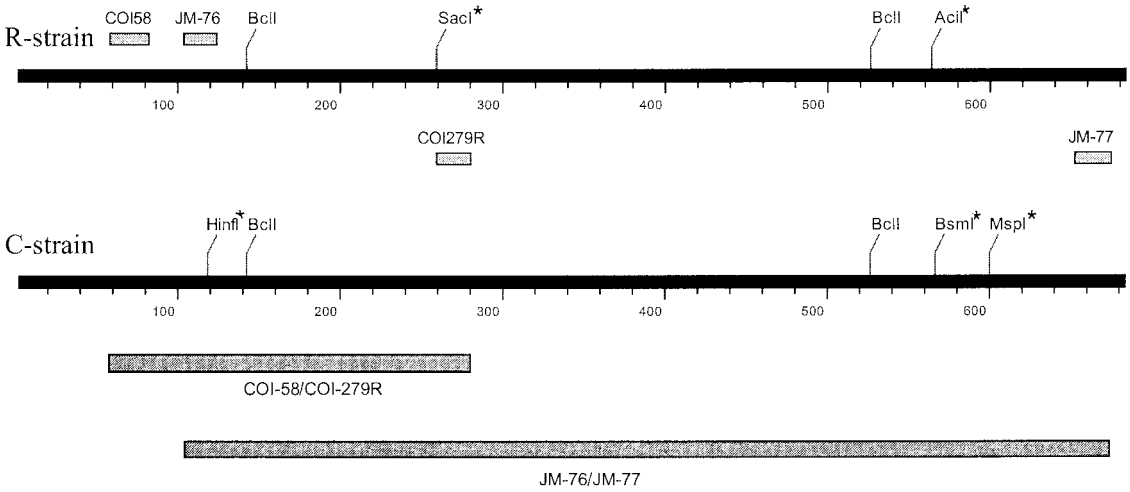

Fig. 1. Restriction map of a portion of the *COI* gene in fall armyworm mitochondria showing strain-specific restriction sites. JM-76 and COI-58 are forward primers; JM-77 and COI-279R are reverse primers. PCR amplified products from specific primer pairs are presented below the maps. Strain-specific sites are indicated by an asterisk.

manufacturer's instructions. The DNA preparation was increased to a final volume of 50 μl with each PCR reaction using 1 μl (between 0.1 and 0.5 μg).

PCR-RFLP Analysis. PCR amplification of the mitochondrial *COI* gene was performed in a 40-μl reaction mix containing 4 μl of 10× reaction buffer, 1 μl of 10 mM dNTP, 0.5 μl of 20 μM primer mix, 1 μl of DNA template, and 0.5 μl of *Taq*DNA polymerase (New England Biolabs, Beverly, MA). The thermocycling program was 94°C (1 min), followed by 33 cycles of 92°C (45 s), 56°C (45 s), 72°C (1 min), and a final segment of 72°C for 3 min. The appropriate restriction enzyme was diluted in 1× reaction buffer to a concentration of 1 U/μl, and 5 μl was added to 8 μl of each PCR reaction. Digestions were at 37°C for 1 to 2 h. Two microliters of 6× gel loading buffer was added to each sample, which was run on a 1.8% PCR grade agarose (Fisher, Hampton, NH) horizontal gel. Typically, 40 or 96 PCR amplifications and restriction digests were performed at the same time by using either 0.2-ml tube strips or 96-well microtiter plates. Primers were synthesized by Integrated DNA Technologies (Coralville, IA), and those for the *COI* region included the pairs *JM76* [5′-GAGCTGAATTAGG(G/A)ACTCCAGG-3′] and *JM77* [5′-ATCACCTCC(A/T)CCTGCAGGATC-3′] for the *COI* region (Levy et al. 2002) and *COI-58* (5′-GGAATTTGAGCAGGAATAGTAGG-3′) and *COI-279R* (5′-CCTGATATAGCTTTCCCACG-3′). Primers for the *ND1* gene were *FAW16S* (5′-TTCAAACCGGTGTAAGCCAGG-3′) and *FAWND1* (5′-TAGAATTAGAAGATCAACCAG-3′) (Prowell et al. 2004).

Results

Characterization of Potential Strain-Specific Polymorphisms. Comparisons among different published sequences of the mitochondrial COI gene from representatives of the R-strain and C-strain revealed several base pair differences, some of which were associated with altered restriction sites (Fig. 1). Three of these polymorphic restriction sites, for BsmI, SacI, and AciI, are located in portions of the fragment amplified by the *JM-76/JM-77* primers that produce bands readily identified by agarose gel electrophoresis (Fig. 2). To determine whether the presence of these sites correlated with strain-specific mitochondrial lineages, we examined fall armyworm samples from several locations in southern and central Florida for their marker combinations.

Strain specificity was generally observed for the BsmI, SacI, and AciI sites as determined by a comparison with the diagnostic MspI marker (Table 2). The *JM-76/JM-77* PCR fragment from nearly all samples (58/59) identified as the R-strain by the absence of the MspI site were digested into two bands by either AciI or SacI, whereas the presence of the MspI site was associated with the absence of the AciI and SacI sites (68/68), indicating that these sites are limited to and identify the R-strain lineage. In the one exception, an R-strain individual (lacking the MspI site) also lacked the AciI site. A similar but converse relationship was observed with the BsmI site, which is virtually always linked to the C-strain-specific MspI site (124/125), making it a marker of the C-strain. Again, there was an exceptional R-strain sample that carried the BsmI site.

The sequence comparisons also identified a HinfI site that is located near one end of the *JM-76/JM-77*-amplified region, requiring a separate set of primers for its efficient detection. The region of the HinfI polymorphic site is contained within the 220-bp fragment amplified by the *COI-58/COI-279R* primer combination, producing bands of 60- and 160-bp if present (Fig. 2B). A nearby BclI site is present in both strains and was used as a positive control for restriction enzyme activity. Like the BsmI marker, the HinfI site was

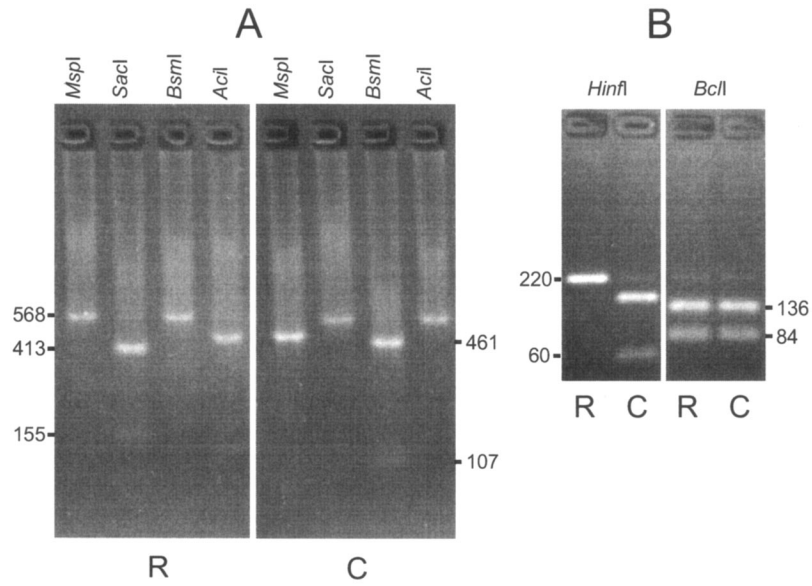

Fig. 2. Agarose gels displaying strain-specific RFLPs from the COI gene. (A) PCR-amplified fragment produced by the JM-76/JM-77 primer pair and cut with the specified restriction enzyme. The uncut fragment is 568 bp. Samples were fractionated on a 1.8% agarose horizontal gel. (B) PCR-amplified fragment produced by the COI-58/COI-279R primer pair and digested with the specified restriction enzyme. The uncut fragment is 220 bp. Samples were fractionated on a 2% PCR grade agarose horizontal gel. R and C denotes R-strain and C-strain, respectively. Sizes are in base pairs (bp).

nearly always (126/127) correlated with the diagnostic MspI site; therefore, its presence serves as a marker for the C-strain lineage (Table 2). In the two exceptional samples, the HinfI site was present, whereas the pattern of the other four restriction sites was diagnostic of the R-strain.

Surveying Populations Outside of Florida. To examine whether the strain-specificity of these markers extended beyond Florida, we tested populations from several other states. Adult males were collected from pheromone traps in Texas, Georgia, and North Carolina and analyzed for the strain-specific restriction sites in the COI gene. Fall armyworm from Georgia and North Carolina are thought to annually derive from populations overwintering in Florida and therefore should be genetically similar. In contrast, it is not

Table 2. Marker combinations observed in specimens isolated from the wild

Location	Sample	Total	MspI	SacI	AciI	BsmI	HinfI	*ND1*
Florida	R-strain	57	−	+	+	−	−	
	C-strain	67	+	−	−	+	+	
	Aberrant R-strain	1	−	+	+	+[a]	−	
	Aberrant R-strain	1	−	+	−[a]	−	−	
	Aberrant C-strain	1	+	−	−	+	−[a]	
	R-strain	168	−					+
	C-strain	104	+					−
Georgia	R-strain	17	−	+	+	−	−	
	C-strain	3	+	−	−	+	+	
	Aberrant C-strain	1	+	−	−	+	−[a]	
North Carolina	R-strain	19	−	+	+	−	−	
	C-strain	2	+	−	−	+	+	
Texas	R-strain	13	−	+	+	−	−	
	C-strain	6	+	−	−	+	+	
	Aberrant C-strain	1	+	−	−	+	−[a]	
Mississippi	R-strain	16	−	+	+	−	−	
	C-strain	21	+	−	−	+	+	
Summary	*COI* agreement	219						
	COI disagreement	5						
	MspI-SacI disagreement	0						
	COI-ND1 agreement	272						
	COI-ND1 disagreement	0						
	Aberrant sites				1	1	3	

[a] Aberrant site.

clear whether Texas fall armyworms interact with those from Florida, and therefore these two populations may be genetically isolated. The majority (60/62) of fall armyworms from all sites showed restriction patterns identical to those found in Florida, demonstrating the general utility of the described strain markers for analyzing North American populations (Table 2). Both exceptions were associated with the absence of the HinfI site (typical of the R-strain), whereas the other four *COI* markers were indicative of the C-strain. The accuracy of the strain-specific COI markers was further indicated by examination of the HinfI restriction site polymorphism in the mitochondrial *ND1* gene that was previously identified with the R-strain and used to analyze populations in several states, the Caribbean, and South America (Pashley 1989, Prowell et al. 2004). In a survey of Florida populations, we found an inverse correlation between this site and the MspI marker in the *COI* gene such that in all samples tested ($n = 285$) the presence of one site was associated with the absence of the other site (Table 2). This indicates that the two polymorphisms are identifying the same mitochondrial lineages.

Rapid Method for Determining Strain Identity. Population studies often require the molecular analysis of large numbers of individual samples. We found the most efficient strategy to accomplish this was to separately digest the fragment produced by PCR amplification by using the *JM-76/JM-77* primer pair with MspI and SacI. The amplified product from the R-strain is cut once by SacI but not by MspI, whereas the C-strain DNA shows the reciprocal pattern. If necessary, the same PCR reaction can be analyzed with AciI or BsmI to confirm strain identity. The utility of this strategy was demonstrated by the analysis of three sample collections from the Mississippi delta region comprised of 1) adult males captured in pheromone traps and stored frozen for several weeks, 2) live larvae from a laboratory colony derived from specimens collected from cotton, and 3) ethanol-preserved larvae collected from egg masses unexpectedly found on royal paulownia trees.

In total, 235 DNA samples were tested in the initial screen of the three collections. In those cases where the PCR amplification was successful ($n = 194$), the MspI and SacI restriction patterns were completely consistent with respect to strain identity, diagnosing 122 samples as R-strain and 72 as C-strain (Table 3). A subset of these ($n = 37$) was tested for the AciI, BsmI, and HinfI polymorphic sites, and in every case the results confirmed the initial assessment (Table 2). We found that collectively through most (June–November) of the cotton growing season 39% (52/133) of the adult males collected from pheromone traps located near cotton fields and successfully tested by PCR were of the C-strain. However, most of these were from the second half of the season (September–November) when the C-strain was the majority (59%; 37/63) captured. This compares with only 21% (15/70) C-strain from June to August. Analysis of the samples from the cotton-derived laboratory colony found that all were of the C-strain, consistent with that strain making up the majority of larvae found on cotton plants. In contrast, all 41 larvae tested representing 12 different egg masses found on neighboring royal paulownia trees were of the R-strain.

Discussion

To enhance current PCR-RFLP methods for determining fall armyworm strains, we have identified several polymorphic restriction sites in the mitochondrial *COI* gene that display the same degree of strain specificity as previously described markers. This information was used to develop a simple method where a single PCR amplification reaction is tested by two restriction enzymes. This strategy eliminates errors in strain identity caused by failed restriction reactions by requiring successful digestion by one enzyme. Furthermore, the same PCR reaction can be tested for two other strain-specific markers without requiring additional amplifications. This potentially increases the accuracy of the procedure, although we did not find that the use of all five markers substantially improved strain identification based on the MspI and SacI patterns.

The utility of these markers for large-scale surveys of fall armyworm populations was demonstrated by assays of almost 200 individual specimens from the Mississippi delta region. Strain identity was successfully obtained in 79% of the samples (153/194) after a single PCR reaction and set of restriction digests. The highest PCR failure rate was typically observed with pheromone trap specimens, not surprising given the number of factors that could potentially degrade DNA quality when samples are exposed to field conditions for several days. Nevertheless, we still found that unambiguous strain identity could be determined in

Table 3. RFLP analysis of three Mississippi fall armyworm sample collections

	$MspI^C$ $SacI^C$ (C-strain)	$MspI^R$ $SacI^R$ (R-strain)	$MspI^R$ $SacI^C$ (R-hybrid)	$MspI^C$ $SacI^R$ (C-hybrid)	No data
Washington Co., MS (pheromone traps in cotton fields)					
June–Aug.	15	55	0	0	20
Sept.—Nov.	37	26	0	0	15
Washington Co., MS (larvae collected from *P. tomentosa)*	0	41	0	0	6
Laboratory colony (from cotton plants)	20	0	0	0	0
Total	92	122	0	0	41

≈74% of these specimens overall by using our primers and methodology. Given the convenience of pheromone trapping for collecting large numbers of specimens, this failure rate is acceptable and the combination of this collection method with PCR-RFLP provides a powerful strategy for studying host strain population dynamics. For example, we found that almost 40% (52/133) of adult males captured by pheromone traps located near cotton fields were of the C-strain, a proportion similar to that found in sweet corn fields in Florida that are primarily infested by the C-strain (Meagher and Nagoshi 2004, Nagoshi and Meagher 2004). This suggests that the C-strain is a significant, if not the predominant, contributor to fall armyworm infestation in cotton, a conclusion strongly suggested by our finding that a laboratory colony derived from larvae collected from cotton plants is predominantly, if not entirely, C-strain. Furthermore, the increase in the proportion of C-strain captured as the cotton growing season progressed, from 21% (15/70) in June–August to 59% (37/63) in September–November, was consistent with cotton preferentially supporting the development of one or more generations of this strain.

The extensive number of egg masses observed on royal paulownia trees in the same county as the pheromone traps located near cotton fields was unexpected because they are not known to be a host of fall armyworm. Because these infestations occurred during the period when high numbers of the C-strain were being capture by pheromone traps, we thought it likely that the royal paulownia specimens were derived from the same populations. This turned out to not be the case because all those adults tested from royal paulownia were of the R-strain. Therefore, it seems that the increase in the numbers of R-strain in the Mississippi delta region approximates that of the C-strain despite the differences in plant hosts. A more detailed comparison of the population dynamics of the two strains could provide important information on migration behavior and help identify those factors promoting both populations. The results also suggest substantial strain specificity in oviposition preference with respect to royal paulownia, understanding the details of which could provide insight into how females choose their plant hosts for depositing egg masses.

Several laboratories have used molecular markers to examine strain populations (Pashley et al. 1985; Pashley 1986, 1989; Lu et al. 1992; Pashley and Ke 1992; Lu and Adang 1996; Prowell et al. 2004). The *ND1* mitochondrial marker was used to examine populations across a broad geographical range, including those in Louisiana, Florida, Puerto Rico, Guadeloupe, and French Guiana (Prowell et al. 2004). The capacity of this mitochondrial haplotyping to distinguish between strains was equivalent to that exhibited by other methods, including amplified fragment length polymorphism and esterase allozyme genotyping, legitimizing its use as a strain marker. The MspI polymorphism in the *COI* gene was initially shown to be strain specific by the examination of several strain-specific fall armyworm colonies generated from populations in Mississippi, Louisiana, and Georgia and was later used for more extensive studies of Florida populations (Lu et al. 1992, Lu and Adang 1996, Meagher and Nagoshi 2004). Our direct demonstration that the two markers identify the same mitochondrial lineages confirms the legitimacy of comparisons between these studies despite their use of different molecular criteria to identify strains.

The more detailed characterization of the mitochondrial *COI* gene should be useful in differentiating fall armyworm specimens from other lepidopteran species that are morphologically similar. Even closely related species will differ in the restriction sites present in the *COI* gene, with the differences becoming more apparent as more sites are analyzed. Although our anecdotal observations suggest that the pheromone trapping methods used in this study are very specific to fall armyworm, at least with respect to Florida populations, substantial nontarget captures were found using pheromone lures in the northeastern United States (Fleischer et al. 2005). We anticipate that DNA from a species other than fall armyworm should either not generate a PCR product because of divergence within the primer sequences or the amplified fragment will differ in restriction sites. The latter event is rare because of 224 samples characterized for strain identity by the five strain-specific restriction sites in the *COI* gene, only five (2%) samples gave patterns inconsistent with either strain, and these samples were aberrant at only one site (Table 1). The proportion of samples that yielded no PCR product was higher, ranging from 5 to 20% overall. However, many, if not most, of these samples are likely to result from poor quality sample DNA, and regardless, they do not lead to a strain identification. These results indicate that errors in strain identity caused by contamination by non-fall armyworm species are generally inconsequential. The characterization of the *COI* gene from other Lepidoptera should provide better molecular tools for identifying different species and for determining the specificity of different trapping and collection methods in different areas.

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