FR Tandem-Repeat Sequence in Fall Armyworm (Lepidoptera: Noctuidae) Host Strains

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ABSTRACT The fall armyworm, *Spodoptera frugiperda* (J. E. Smith), consists of two sympatric strains (rice and corn) that are morphologically identical. To facilitate strain identification, a polymerase chain reaction (PCR)-based method was developed to detect *FR*, a reportedly strain-specific element found in the nuclear genome in large tandem-repeat clusters. The efficacy and accuracy of the PCR method were tested on inbred laboratory lines of both rice and corn strains. Although it was previously thought that *FR* sequences were exclusive to the rice strain, we were able to isolate homologous sequences in a subset of the corn strain population. Even in these cases, the PCR method detected strain-specific amplification patterns, indicating that the distinction between rice and corn strains is caused by differences in copy number and organization in the genome rather than simply the presence or absence of *FR*. A second polymorphism detectable by PCR was also found that distinguishes between rice and corn strain laboratory colonies. The availability of PCR-based methods for detecting strain- and population-specific molecular markers will facilitate studies on strain distribution in the field and the examination of strain-specific behavior.

KEY WORDS *Spodoptera frugiperda*, strain identification, *FR* sequence

THE FALL ARMYWORM, *Spodoptera frugiperda* (J. E. Smith), is a major agricultural pest of corn, forage grasses, and rice, and an occasional pest of many other crops, including cotton and peanuts. Two sympatric strains have been identified that differ in their preference of plant host (Pashley 1986). The rice strain is associated with rice and Bermuda grass, while the corn strain displays a preference to corn and cotton, although this host specificity is not completely exclusive (Pashley 1988a). The two strains also differ in physiological characteristics, including rates of development and larval and pupal weights (Pashley 1988b, Quisenberry et al. 1988, Whitford et al. 1992, Whitford et al. 1988), and display differences in plant host ovipositional preference and mating preference that suggest possible limitations in interstrain mating (Pashley et al. 1992, Whitford et al. 1988). However, because they are morphologically indistinguishable, extensive and conclusive information about the distribution of and behavioral interactions between these strains in the wild has been difficult to obtain, although such data are critical for any comprehensive fall armyworm control program.

The rice and corn strains can be distinguished by several restriction fragment-length polymorphisms in mitochondrial DNA (mtDNA), as analyzed by Southern blot analysis or direct examination of stained agarose gels (Lu and Adang 1996, Pashley 1989). Lu and Adang (1996) demonstrated that digestion with *Msp*I generates particularly distinctive strain-specific mtDNA restriction patterns. This observation led to the development of a polymerase chain reaction (PCR)-based method in which a portion of the mitochondrial cytochrome oxidase subunit I (COI) gene that contains one polymorphic *Msp*I site is amplified (Levy et al. 2002). This procedure makes large-scale field studies practical, as it can be performed using only a few nanograms of DNA isolated from individuals sampled over many days (R. Meagher and M. Gallo-Meagher, personal communication).

Another strain-specific marker from the nuclear genome is a repeated DNA sequence called *FR* (for fall armyworm Rice strain). Southern blot hybridization has shown the *FR* sequence to be present extensively in the rice strain, but absent in corn strain (Lu et al. 1994). The 189-bp *FR* unit is similar to satellite DNA sequences in being A-T rich and undermethylated, accounting for an estimated 0.05% of the genomic DNA. It is organized in large tandem repeat arrays and is sex specific in abundance, showing an ≈120-fold reduction in copy number in males. This sex specificity suggests that *FR* may be most abundant on the *Y*-chromosome, which in Lepidoptera is carried in the female (Lu et al. 1994). The availability of a strain-specific nuclear polymorphism to complement the mitochondrial marker makes it possible to address issues concerning strain behavior and interactions.

For example, if a significant fraction of wild individuals carries a hybrid marker combination (i.e., the mtDNA restriction fragment-length polymorphism specific to one strain combined with an *FR* distribution specific to the other), it would indicate the occurrence of substantial interstrain mating, a phenomenon for which there is limited and conflicting experimental evidence (Pashley and Martin 1987, Whitford et al. 1988, Adamczyk et al. 1996, Prowell 1998, McMichael and Prowell 1999). However, the need to use Southern blot analysis to detect *FR* is a potential limitation, as the quality of DNA from field-collected samples is often compromised.

In this study, we describe a rapid PCR-based method for detecting large tandem-repeat clusters of *FR* sequences from individual adult moths. An advantage of this procedure is that the DNA from a single individual can be analyzed multiple times by PCR for different molecular markers. In addition, the increased sensitivity of PCR over conventional Southern blot analysis allowed detection of *FR* in corn strain populations. This indicates that the strain specificity of *FR* sequences involves the number of copies and their organization into tandem repeat clusters, rather than the simple presence or absence of the element.

Materials and Methods

Strains and Collections. Male adult fall armyworms were collected from field traps and laboratory-reared colonies. The *Tifton* corn strain colony was obtained from the United States Department of Agriculture-Agricultural Research Service (USDA-ARS, Tifton, GA). The *Ona* colony originated from larvae isolated from grass pastures from the Range Cattle Research and Education Center (Ona, FL), and has been maintained by inbreeding for almost 1 yr. The *Krome* colony was established from larvae isolated from corn plants near Homestead in Miami-Dade County, Florida, and has been in culture for 6 mo. Fall armyworms were reared on a pinto bean-based artificial diet (Guy et al. 1985) and cultured, as previously described (R. Meagher and M. Gallo-Meagher, personal communication).

DNA Preparation. Individual adult fall armyworm were homogenized in 1 ml of homogenization buffer (0.03 M Tris-HCl at pH 8.0, 0.1 M NaCl, 0.2 M sucrose, 0.01 M ethylenediaminetetraacetic acid (EDTA) at pH 8.0, 0.5% Triton X-100) in a 5-ml Dounce homogenizer using either a hand pestle or a motorized mixer. To remove large debris, the homogenate was filtered through a 5-ml plastic syringe plugged with cheese cloth (prewet with distilled water) into a 1.5-ml microfuge tube. The Dounce homogenizer was washed with 800 μl of buffer, which was filtered and added to the homogenate. Cells and nuclei were pelleted by centrifugation at 12,000 $\times$ *g* for 10 min at 4°C, and the supernatant was removed by aspiration. The pellet was resuspended in 600 μl nuclei buffer (0.01 M Tris-HCl at pH 8.0, 0.35 M NaCl, 0.1 M EDTA, and 1% *N*-lauryl sarcosine), and extracted with 400 μl phenol-chloroform (1:1). The supernatant was transferred to a new 1.5-ml tube, precipitated with 400 μl isopropanol for 1 h at room temperature, and centrifuged at 12,000 $\times$ *g* for 10 min. The DNA pellet was washed with 70% ethanol and dried. The pellet was resuspended in 50 μl distilled water, followed by purification using DNA Clean and Concentrator-5 columns (Zymo Research, Orange, CA), according to manufacturer's instructions. Each PCR reaction used 1 μl of the DNA preparation (between 0.1 and 0.5 μg).

PCR Analysis. All genomic DNAs used in this study were tested by PCR for the mitochondrial COI gene restriction fragment-length polymorphism to confirm strain identity. PCR amplification of genomic DNA was performed in a 50-μl reaction mix containing 5 μl 10$\times$ reaction buffer with $MgCl_2$ (Promega, Madison, WI), 1 μl 10 mM dNTP (New England Biolabs, Beverly, MA), 0.5 μl 20 μM primer mix, 1 μl DNA template, and 0.5 μl *Taq* DNA polymerase (Promega, Madison, WI). Amplification of the COI gene used primers *JM76* and *JM77* and began with an initial incubation at 94°C (1 min), followed by 35 cycles of 94°C (1 min), 56°C (1 min), 72°C (1 min), and a final segment of 72°C for 5 min. Upon completion of the PCR, 0.5 μl of *Msp*I was added to each reaction and incubated at 37°C for 1 h. A total of 5 μl of gel loading buffer was added to each sample, and 20 μl was loaded on a 1.5% agarose gel. Amplification of the *FR* and other fragments (unless otherwise noted) used an initial incubation at 94°C (1 min), followed by 38 cycles of 94°C (45 s), 60°C (45 s), 72°C (1 min), and a final segment of 72°C for 5 min. A total of 5 μl of gel loading buffer was added to each sample, and 20 μl was loaded on a 2% agarose gel.

Primers were synthesized by DNAgency (Malvern, PA). They included *JM76* (5′-GAGCTGAATTAGG(G/A)ACTCCAGG-3′) and *JM77* (5′-ATCACCTCC(A/T)CCTGCAGGATC-3′), which span the mitochondrial cytochrome oxidase C subunit I gene (COI). The *FR* primers are from the *FR* sequence and include *FR-a* (5′TTTTACACCGGTCACAACGA-3′), *FR*-c (5′-TCGTGTAAAACGTACTTTCTT-3′), and *FR*-2 (5′-GACATAGAAGAGCACGTTT-3′). Primers specific for fragment c3 are *CoA32* (5′-CACGACTGGAAACTACCTGA-3′), *CoA464* (5′-TGTAGCCTGATCCGAAGC-3′), *CoB22* (5′-TCACACCACAATCACAATAA-3′), and *CoB478* (5′-TTCCTTCTCCTGCTTTGA-3′).

Cloning of PCR Fragments. PCR fragments were isolated from agarose gels using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA). PCR fragments were subcloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and selected by X-Gal staining, according to manufacturer's instructions. The appropriate subclones were identified by restriction enzyme analysis. For each individual, at least one subclone was randomly chosen for DNA sequencing. DNA sequencing was performed by au-

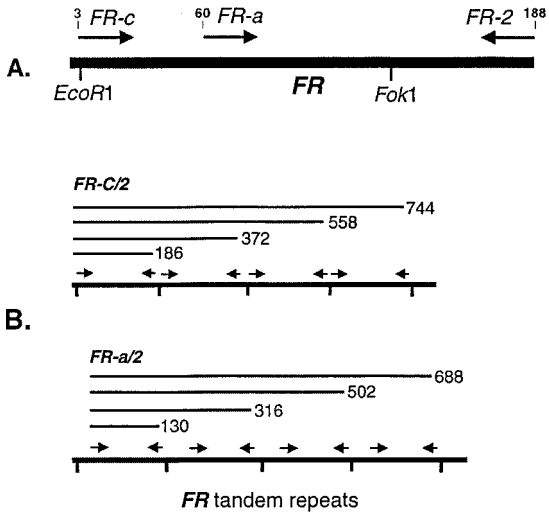

Fig. 1. Map showing the location of *FR*-specific primers and predicted PCR products. (A) Location of primers (arrows) within a single *FR* unit. Locations of *Eco*RI and *Fok*I sites are noted. (B) Subset of expected PCR products produced by the primer combinations *FR-c*/*FR-2* and *FR-a*/*FR-2* if *FR* sequences are organized in large tandem-repeat arrays. Numbers indicate predicted sizes (bp).

tomated sequencers at the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, FL).

Results

The *Tifton* and *Ona* laboratory colonies were initiated from and supplemented with larvae isolated from either corn or grass hosts, respectively. PCR analysis using primers (*JM76*/*JM77*) that detect the strain-specific mtDNA marker demonstrated that the great majority of *Tifton* individuals (29 of 30) are of the corn strain, while *Ona* is primarily rice strain (15 of 15). Several primer combinations derived from the consensus *FR* sequence were used to PCR amplify DNAs from *Ona* and *Tifton* individuals whose strain identities were confirmed by PCR analysis of the mtDNA marker. Two primer combinations were found to consistently produce strain-specific amplification patterns (Fig. 1A). The primer pair *FR-c*/*FR-2* anneal near the ends of the *FR* sequence and are predicted to amplify a 186-bp fragment from *FR*. Primer *FR-a* anneals more internally and, in combination with *FR-2*, should amplify a 130-bp fragment from a single *FR* unit. However, because *FR* sequences are arranged in large tandem repeat clusters (Lu et al. 1994), they can be amplified in more complex patterns that will produce higher molecular weight bands of predictable sizes (Fig. 1B). This prediction was tested by PCR amplification of rice strain DNA with either *FR-c*/*FR-2* or *FR-a*/*FR-2* primer combinations. In both cases, a higher molecular weight smear was observed within which a DNA ladder is seen with fragment sizes consistent with the amplification of multiple *FR* units (Fig. 2A). That this ladder was derived from repeated *FR* units was confirmed by digestion of the

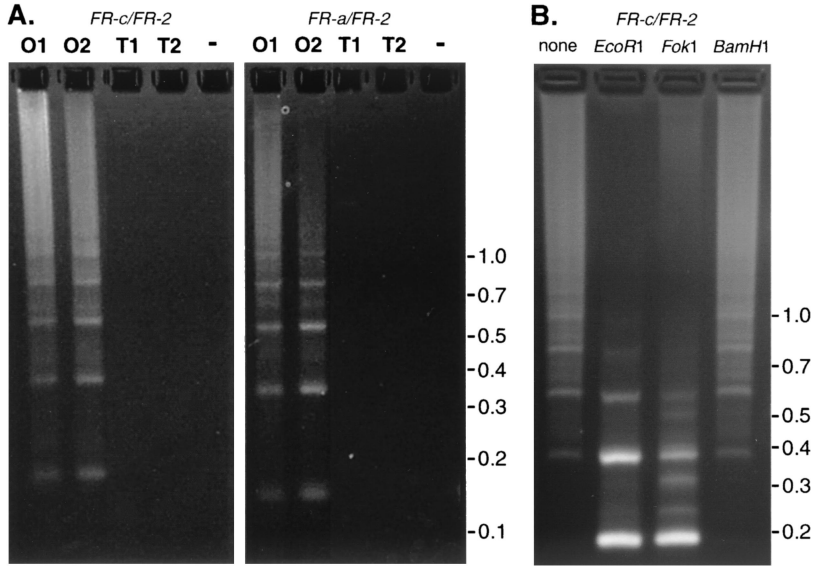

Fig. 2. Ethidium bromide-staining pattern of PCR products from *Tifton* (T) and *Ona* (O) individuals representing corn strain and rice strain, respectively. (A) PCR amplification using primers *FR-c*/*FR-2* or *FR-a*/*FR-2* on DNA from two *Ona* (O1–2) and *Tifton* (T1–2) females using an annealing temperature of 60°C. Similar results were obtained with males (data not shown). Higher molecular weight ladder is only present with rice strain DNA. (-) Denotes control reaction lacking template DNA. (B) PCR amplification products of *Ona* female DNA (from one individual) undigested (none), or digested with *Eco*RI, *Fok*I, or *Bam*HI. Sizes are in kilobases.

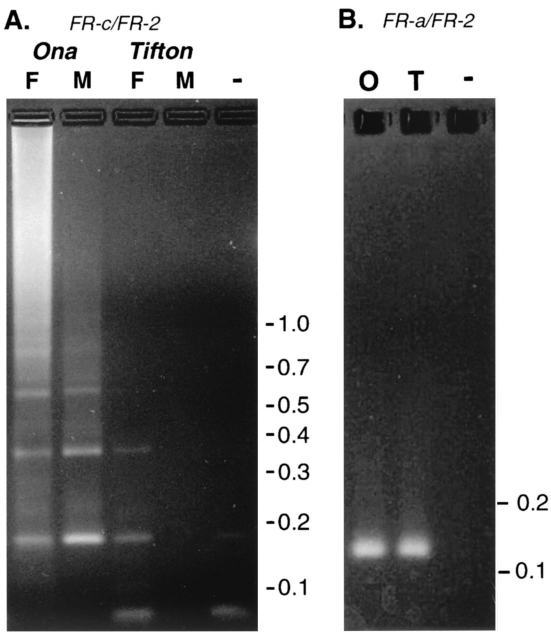

Fig. 3. PCR amplification of fall armyworm DNA using *FR* primers. (A) Primers *FR-c/FR-2* were used at an annealing temperature of 56°C to amplify *Ona* and *Tifton* female (F) and male (M) DNA. Faint bands are occasionally in the *Tifton* reactions. (B) Amplification using primers *FR-a/FR-2* of isolated fragment from *FR-c/FR-2* PCR reaction. Both *Ona*- and *Tifton*-derived fragments produce the *FR-a/FR-2* band at the expected size (120 bp). Sizes are in kilobases. (-) Denotes control reaction lacking template DNA.

PCR products using selected restriction enzymes. *Eco*RI and *Fok*I both have a single site within the consensus *FR* sequence (Fig. 1A). Digestion with either enzyme collapses the DNA ladder such that most of the DNA is now present in lower molecular weight bands with sizes consistent with fragments containing 1–2 *FR* units (Fig. 2B). In contrast, digestion with *Bam*HI, which does not cut *FR*, has no effect on the PCR pattern.

Further confirmation that *FR* sequences are being amplified came from DNA sequence analysis. Genomic DNA was isolated from individual *Ona* adults and PCR amplified using primers *FR-c/FR-2.* DNA of ≈184 bp was isolated and reamplified with primers *FR-a/FR-2.* This second amplification produced a band of ≈120 bp (Fig. 3B), which was then cloned and sequenced. When DNA from two *Ona* individuals was tested in this way, the sequences differed from the FR consensus at no more than three sites (Fig. 4). These results confirm that *FR* sequences are present in our laboratory rice strain colony and can be amplified by our primers.

In contrast to the *Ona* results, PCR amplification of genomic DNA from the *Tifton* corn strain did not produce a DNA ladder (Fig. 2A). This would be consistent with the absence of large *FR* clusters in this population. However, at lower annealing temperatures, a subset of the *Tifton* samples did occasionally produce PCR bands that were not present in control reactions and comigrated with a portion of the *Ona* DNA ladder (Fig. 3A). It is therefore possible that *FR* sequences are present in the corn strain, but are not arranged in large tandem arrays

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FR consensus        TATTCGTGATTGCACTTCCACTACAATCTGTTTCACGGATGAGTTGAAGG 50
Ona1          (FR-a)·····T·····C·····
Ona2          (FR-a)·····T·····C·····
Tifton1a      (FR-a)·····T·····C·····
Tifton1b      (FR-a)·····T·····C·····C·····
Tifton2a      (FR-a)·····T·····C·····
Tifton2b      (FR-a)·····T···C·····C·····
Krone1        (FR-a)·····C·····C·····
Krone2        (FR-a)·····T·····C·····

FR consensus        AAATATCG----GATAAGCATTCTTTGTCGGAAATCATAATAT 89
Ona1          ·····---·····G·····G·(FR-2)
Ona2          ·····---···G·····C·(FR-2)
Tifton1a      ·····---···C·····T·(FR-2)
Tifton1b      ·····---···T·····(FR-2)
Tifton2a      ·····---···G·····C·(FR-2)
Tifton2b      ·····---·····(FR-2)
Krone1        ·····GATT·····(FR-2)
Krone2        ·····---···G·····C·(FR-2)
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Fig. 4. Sequence comparison between the consensus *FR* sequence and the *FR-a/FR-2* PCR-amplified fragments from rice strain (*Ona*) and corn strain (*Tifton* and *Krome*) individuals. In each case, DNA from two individuals were isolated, amplified, and sequenced. Two clones were sequenced from each *Tifton* individual. Shown is the 89-bp region that is spanned by primers *FR-a* and *FR-2*. The dots (·) indicate identity to the consensus *FR* sequence, while dashes (-) indicate region of the GATT insertion in the *Krome2* sequence.

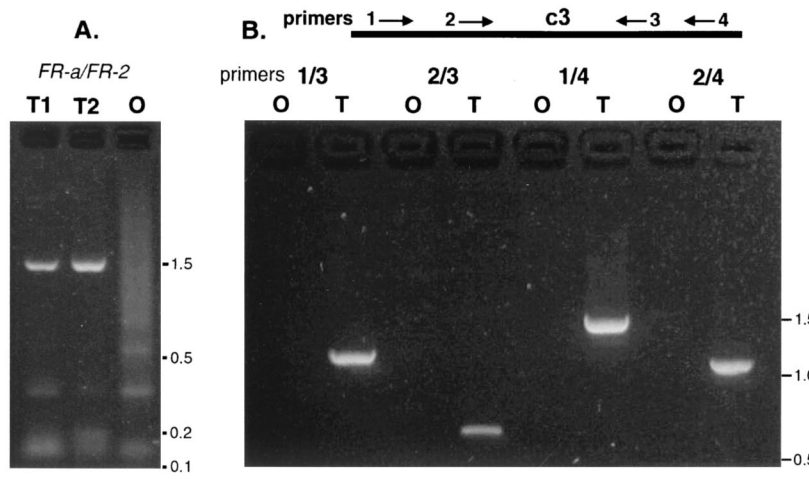

Fig. 5. PCR-amplified bands produced by primers *FR-a/FR-2* at 45°C annealing temperature. (A) Ethidium bromide-staining pattern of PCR products from two *Tifton* and one *Ona* individual. The 1.5-kb band (designated c3) was isolated for further study. (B) Locations of primers are depicted with respect to fragment c3. In the ethidium bromide-stained gel, only DNA from the *Tifton* (T) individual results in the predicted bands. No amplification is seen with DNA from the *Ona* (O) individual. Sizes are in base pairs. Primer symbols: 1 = *CoB22*; 2 = *CoB478*; 3 = *CoA464*; 4 = *CoA32*. Sizes are in kilobases.

or are of degenerate sequence. To address this possibility, we identified two *Tifton* samples whose corn strain identities were confirmed by analysis of the strain-specific mtDNA marker, and that produced an ≈184-bp band when PCR amplified with *FR-c/FR-2*. The isolated fragment was reamplified with *FR-a/FR-2*, producing a 120-bp fragment that was isolated and cloned (Fig. 3B). Two independent clones from each individual were sequenced, and all showed strong homology to the consensus *FR* (Fig. 4). We used the same procedure to analyze amplified sequences from another laboratory colony (*Krome*) derived from larvae found on corn hosts and carrying the corn strain-specific mtDNA marker (six-sixths tested). Once again, *FR*-like sequences were isolated, confirming that these elements are not exclusively limited to rice strain populations (Fig. 4). Therefore, it appears that the strain specificity with respect to *FR* has more to do with differences in the number and organization of *FR* units, rather than the presence or absence of the sequence.

During our studies with *FR-a/FR-2*, we found that an ≈1.5-kb fragment (called c3) was produced from *Tifton* DNA when an annealing temperature of 45°C was used that was not observed with *Ona* or control reactions (Fig. 5A, data not shown). We cloned c3 and sequenced 451 and 743 bp from either end. The sequence analysis showed that the c3 fragment was amplified by *FR-a* alone, and that the sequenced region showed no significant homology to *FR* (in a pairwise BLAST comparison) nor to any entry in the National Center for Biotechnology Information sequence databases. Four primers more specific to c3 were generated from sequences internal to the *FR-a* priming sites. Four different pairwise combinations of the c3-specific primers were tested, and each generated a single band with *Tifton* DNA, but not with *Ona* (Fig. 5B). To test the specificity of the c3 polymorphism to the *Tifton* colony in particular and to corn strain in general, we analyzed several individuals from the *Tifton*, *Krome*, and *Ona* colonies using primers *CoB478/CoA32*. All *Tifton* samples ($n = 9$) produced the c3-derived fragment, as did most of the *Krome* samples (5 of 7). In contrast, the polymorphism is rare in *Ona* (1 of 12). These data indicate that the distribution of the c3 polymorphism varies by population and perhaps also by strain, making it a potentially useful marker for identifying different fall armyworm genotypes.

Discussion

Our isolation of *FR* sequences from two corn strain populations conflicts with a previous report (based on Southern blot analyses) that *FR* sequences are present exclusively in the rice strain (Lu et al. 1994). However, given the small size of an individual *FR* unit (189 bp), detecting a small number of copies by Southern blot hybridization would be difficult, particularly if the *FR* units are not clustered. Therefore, it is not surprising that a more sensitive PCR-based method would identify *FR* sequences not previously detected. It is also possible that the corn strain population may be polymorphic for the presence of *FR* sequences, making their detection variable and sample dependent. This latter possibility is likely, given evidence that mating between strains can occur in the laboratory (Whitford et al. 1988), although perhaps in a limited fashion (Pashley and Martin 1987).

In any case, even in those corn strain individuals carrying *FR* sequences, our PCR results showed significant differences compared with rice strain individuals with respect to the number, intensity,

and sizes of the amplified bands. We interpret this as reflecting differences in *FR* copy number and/or their organization in large, tandem-repeat clusters and conclude that this PCR-based method provides a rapid means of detecting these differences. In a preliminary screen of our laboratory colonies, we found that the presence of *FR* clusters correlated with the diagnostic strain-specific polymorphism in the mitochondrial COI gene (Levy et al. 2002). DNA ladders were absent in all individuals of the corn strain (12 of 12) and present in the majority of rice strain samples (12 of 15). Therefore, there is a good, although not perfect, correlation between the mitochondrial marker and *FR* sequences. The discrepancy, while small, is interesting in that it suggests that cross-hybridization may be occurring in the population from which the *Ona* colony was derived, and that the resulting hybrids can be detected by PCR-based analysis of these molecular markers. We are currently testing these possibilities.

While our data do not allow a determination of the number of *FR* sequences present in the corn strain genome, the different sequences isolated from *Krome* and *Tifton* individuals suggest multiple *FR* copies. Comparisons among the *Ona*, *Tifton*, and *Krome* sequence data also uncovered no evidence of strain-specific sequence polymorphisms. For example, one rice strain sequence (*Ona2*) was identical to two corn strain sequences (*Tifton2b* and *Krome2*), each differing from the *FR* consensus at the same three sites. Lu et al. (1994) observed that sequence divergence between *FR* repeats is the lowest reported for tandem repeats in insects. Our comparison of rice and corn strain *FR*s is consistent with that conclusion.

The presence of two strains of fall armyworm with significant physiological and behavioral differences, but that are morphologically identical, complicates efforts to control this agricultural pest and understand its biology. Remarkably little is known about whether and to what degree interstrain matings occur in the wild, how the resulting hybrids differ from the parental strains, and whether there are strain-specific differences in migration and overwintering behaviors. Our PCR-based method for detecting *FR* clusters makes possible the rapid analysis of large numbers of field-collected samples. When used in combination with other PCR-detectable polymorphisms, such as the population-specific c3 fragment (Fig. 4) and the strain-specific restriction fragment-length polymorphism in the COI gene, both nuclear and mitochondrial strain-specific molecular markers can be identified and compared. These methods are the basis of an ongoing study examining the distribution and behavior of the fall armyworm strains in the wild.

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