

Differences in Amplified Fragment-Length Polymorphisms in Fall Armyworm (*Lepidoptera: Noctuidae*) Host Strains

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ABSTRACT Amplified fragment-length polymorphic (AFLP) loci were examined in the fall armyworm, *Spodoptera frugiperda* (J. E. Smith), to assess their ability to distinguish 2 host-associated strains. Cluster analyses of variation at 10 AFLP loci amplified with 5 primer pairs revealed 2 groups. Each group contained a majority of individuals from 1 or the other host-associated strain as defined by habitat and mtDNA type. Discordance between habitat/mtDNA strain assignments and AFLPs occurred more frequently in the rice strain (8%) than in the corn strain (3%), and at a frequency of 5% overall. Results indicate that AFLPs exhibit strain-specific variation comparable to that of mtDNA and allozymes. In combination with other markers, AFLP loci will be useful for developing a system for identification of fall armyworm strains and hybrids.

KEY WORDS *Spodoptera*, host-strains, hybridization, polymorphisms, speciation

ADVANCES IN DNA technology made during the past 3 decades have revolutionized the application of molecular genetic techniques to studies in evolutionary biology. Comparison of DNA sequences at different taxonomic levels has allowed evolutionary biologists to identify lineages, propose evolutionary relationships, and test hypotheses about patterns of evolution and speciation. In addition, the ability to assign genetic characters to distinct evolutionary entities has allowed identification of interspecific hybrids and the ensuing introgression of genes and traits. Despite the difficulties of inferring process from pattern, model systems exist that provide guidelines for the use of genetic markers in evolutionary biology (Harrison 1991, Templeton 1994).

Host-associated genetic differentiation in fall armyworm, *Spodoptera frugiperda* (J. E. Smith), is a model system for studying traits that differ between species. Two strains have been identified that appear to be in the early stages of speciation (Pashley 1986, Prowell 1998). Life-history characteristics and molecular data suggest fall armyworm strains are more likely to be host-associated sibling species than host races or panmictic, polymorphic populations (Diehl and Bush 1984; Pashley 1988b, 1989, 1993; Prowell 1998). Strains are sympatric as well as synchronic and tend to use different hosts, although host fidelity is not complete in either strain (Pashley 1993). Strains exhibit genetic differentiation in 5 allozyme loci (Pashley 1986), mitochondrial DNA (mtDNA) haplotypes (Pashley 1989, Lu and Adang 1996), nuclear DNA restriction fragment-length polymorphisms (RFLP) (Lu et al. 1992), and repeated DNA sequences (Lu et al. 1994). Other trait differences include development associated with performance on each host (Pashley et al. 1987a, Pashley 1988a, Whitford et al. 1988), responses

to insecticides (Pashley et al. 1987b), and temporal mating activities (Pashley et al. 1992). Two traits with significant strain-biased expression, esterase and mating time, are X-linked (Pashley et al. 1992, Prowell 1998). This finding is consistent with the recently recognized phenomenon in *Lepidoptera* that a disproportionate number of traits distinguishing closely related species are X-linked (Sperling 1994, Prowell 1998).

The extent to which the 2 strains interbreed is equivocal. Although an initial laboratory study suggested a unidirectional behavioral barrier to inter-strain mating (Pashley and Martin 1987, Pashley 1993), others have not (Whitford et al. 1988; unpublished data). In cross attraction studies conducted in the field, males of both strains preferred females of their own strain more often but were nevertheless attracted to females of both strains (Pashley et al. 1992). Whether mating results when cross attraction occurs has not been determined. Interstrain differences in mating times, coupled with preferential intrastrain mating, probably result in assortative mating. However, all potential reproductive isolating factors exhibit some degree of overlap between strains, which could lead to a limited degree of hybridization. In addition, fall armyworms spend some proportion of adult life in nonhost environments foraging for nectar, providing additional mating opportunities (Sparks 1979, Prowell 1998).

To address the issue of hybridization, an unambiguous identification system for strains and hybrids based on allozymes, mtDNA, and nuclear DNA markers has been sought. The composite distribution of allozyme and mtDNA genotypes has not provided sufficient resolving power to discriminate between interstrain hybridization and genetic overlap (ances-

tral polymorphisms) (Pashley 1989). Nuclear markers examined to date, RAPDs, ITS1 sequences (D.P.P., unpublished data), and a sodium channel intron sequence (Adamczyk et al. 1997), revealed intrastain variability but no strain-specific differences.

In this study, we report on the use of amplified fragment-length polymorphisms (AFLP) as potential markers for fall armyworm strains. AFLPs have greater potential for uncovering diagnostic differences than many molecular markers because of the vast number of loci available for study. Theoretically, >4,000 pairs of primers with 3 nucleotide extensions alone can be examined. Markers are distributed randomly throughout the genome, and do not require gene expression for their detection. AFLPs have been used to identify intraspecific varieties in rice (Cho et al. 1996), determine the degree of relatedness between soybean accessions (Maughan et al. 1996), assess genetic variation in endangered plants (Travis et al. 1996), and distinguish morphologically identical *Bacillus anthracis* isolates (Keim et al. 1997). In each case, AFLPs revealed previously undetected levels of variability.

Materials and Methods

Fall Armyworm Samples. Individual fall armyworms were collected as 3rd–5th instars from corn and forage grasses at Louisiana State University Agriculture Center, Ben Hur Research Station, Baton Rouge, LA. mtDNA haplotypes were assayed to make strain assignments (Pashley 1989, Lu and Adang 1996). Thirty-seven individuals from each habitat that possessed the predominant mtDNA haplotype for that habitat were selected for AFLP analysis. We refer to these as the corn (C) and rice (R) strains.

DNA Isolation. The head and thorax of each individual were homogenized in 300 μ l of ice cold 0.25 M sodium citrate in a 1.5-ml microcentrifuge tube with a conical ground-glass tissue grinder, and centrifuged at $3,500 \times g$ for 5 min at 4°C. After transferring the supernatant to a fresh tube, 15 μ l of 0.5 M EDTA (pH 8.0), 10 μ l of 20% SDS, and 10 μ l of 20 μ g/ μ l proteinase K were added to each sample. Samples were inverted gently several times to mix, incubated for 1 h at 60°C, and incubated overnight at 42°C. Samples were centrifuged at $15,800 \times g$ for 10 min at room temperature, and phenol/chloroform extracted. Nucleic acids were precipitated with 2 volumes of -20°C ethanol followed by centrifugation at $3,500 \times g$ for 10 min at 4°C. RNA was removed by digestion with 60 μ l of 0.01 μ g/ μ l RNase A at 37°C for 30 min. RNase was heat inactivated by incubating at 60°C for 30 min. Samples were then allowed to cool slowly to room temperature and stored at 4°C.

Amplified Fragment-Length Polymorphism Sample Preparation. Restriction-ligation protocols followed those of Vos et al. (1995). Approximately 1 μ g of genomic DNA was digested with endonucleases *EcoRI* (Life Technologies, Gaithersburg, MD) and *MseI* (New England Biolabs, Beverly, MA) for 1 h at 37°C in 40 μ l of 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 1 mM dithiothreitol, 0.1 μ g/ml BSA, 5 U *MseI*, and 5 U

Table 1. Adapter and primer sequences (5'-3') used for AFLP analysis in fall armyworm

Primer name	Primer type	Sequence (5'-3')
EcoRI-A1	Adapter	CTCGTAGACTGCGTACC
EcoRI-A2	Adapter	AATTGGTACGCGACTCTAC
EC	+1	GACTGCGTACCAATTCC
ECGA	+3	GACTGCGTACCAATTCCGA
ECGC	+3	GACTGCGTACCAATTCCGC
ECT	+2	GACTGCGTACCAATTCTT
MseI-A1	Adapter	GACGATGAGTCCTGAG
MseI-A2	Adapter	TACTCAGGACTCAT
MA	+1	CATGACTCCTGAGTAAA
MAAG	+3	CATGACTCCTGAGTAAAAG
MACA	+3	CATGACTCCTGAGTAAACA
MACC	+3	CATGACTCCTGAGTAAACC
MAGC	+3	CATGACTCCTGAGTAAACG
MACT	+3	CATGACTCCTGAGTAAACT
MAGG	+3	CATGACTCCTGAGTAAAG

EcoRI. Double-stranded adapters (sequences taken directly from Vos et al. 1995) were ligated by adding to each sample 10 μ l of a mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol, 5 pg *EcoRI* adapters, 50 pg *MseI* adapters, and 1 U T4 DNA ligase (Life Technologies). Ligations were performed for 3 h at 37°C. After ligation, samples were diluted to 500 μ l with 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0), and stored at -20°C .

Amplified Fragment-Length Polymorphism Primers. Adapter-DNA fragments served as templates for selective preamplification with *EcoRI* and *MseI* primers complementary to the adapter/restriction site sequences to which an additional nucleotide had been added (+1 primers). Products from preamplification reactions then served as templates for further selective amplification with *EcoRI* and *MseI* primers complementary to +1 primers to which an additional 1 (*EcoRI*) or 2 (*EcoRI* and *MseI*) nucleotides were added (+2 and +3 primers, respectively; Table 1).

Amplified Fragment-Length Polymorphism Reactions. Preamplification reactions contained 5 μ l (\approx 10 ng) adapted DNA fragments, 20 mM (NH₄)₂SO₄, 50 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 μ M of each +1 primer (EC and MA, Table 1), and 0.5 U Tfl polymerase (Epicentre Technologies, Madison, WI) in a total volume of 20 μ l, and were covered with 25 μ l light mineral oil. Preamplification was performed for 20 cycles with the following profile (Vos et al. 1995): 30 s at 94°C, 1 min at 56°C, and 1 min at 72°C. Successful preamplification was determined by agarose gel electrophoresis of 4 μ l of the preamplification product. The remaining 16 μ l of preamplification product was diluted to 160 μ l with 10 mM Tris-HCl, 1 mM EDTA (pH 8.0), and stored at -20°C .

For selective amplification, *EcoRI* +2 and +3 primers were end-labeled with [γ -³³P]ATP and T4 polynucleotide kinase (Life Technologies) under the following conditions: 1 μ M *EcoRI* primer/ μ l, 70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 100 mM KCl, 1 mM 2-mercaptoethanol, 0.5 μ Ci [γ -³³P]ATP/ μ l, and 0.1 U T4 polynucleotide kinase/ μ l. Selective amplifications were performed in 20 μ l containing 5 μ l of the diluted preamplification product, 0.5 μ M end-labeled *EcoRI*

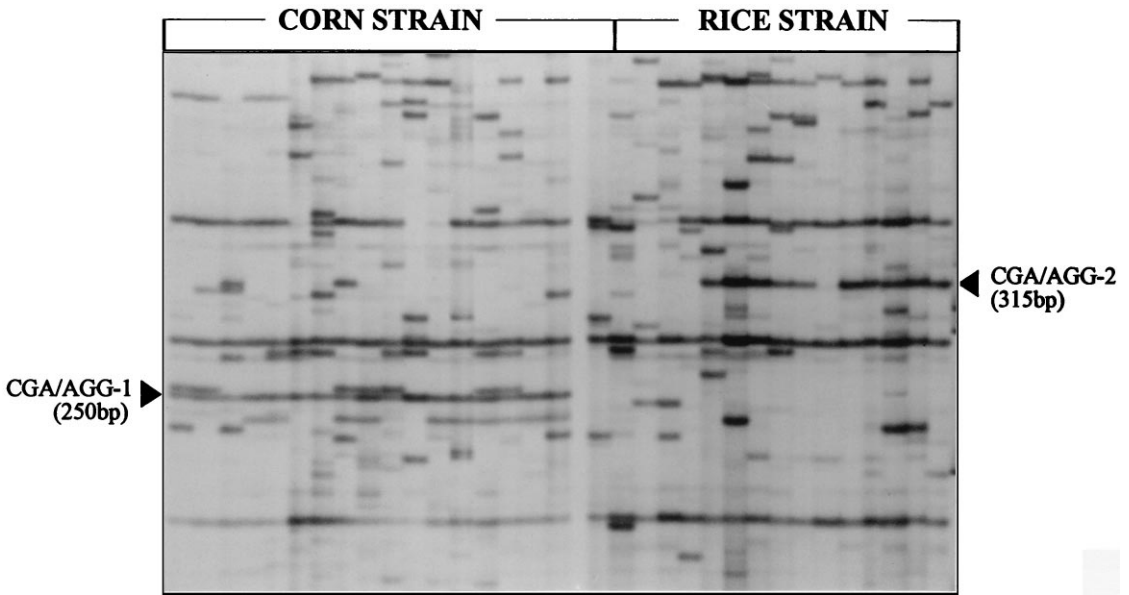


Fig. 1. Two strain-based AFLP loci. Each lane contains the selective amplification products obtained with primers *EcoRI*-CGA (end-labeled with ³³P) and *MseI*-AGG for one sample.

primer, 0.5 μM *MseI* +3 primer, 0.5 U Tfl polymerase, with the same buffer and nucleotide concentrations as in the preamplification reaction. Samples were covered with 25 μl light mineral oil and amplified according to the following Vos et al. profile (1995): 1 cycle of 30 s at 94°C, 30 s at 65°C, 1 min at 72°C; 12 cycles, in which all times and the denaturing and extending temperatures remained the same, and the annealing temperature was decreased by 0.7°C in each cycle; then 23 cycles of 30 s at 94°C, 30 s at 56°C, 1 min at 72°C, ending with 15°C hold. All amplifications were performed in a PTC-100 Programmable Thermal Cycler (MJ Research, Watertown, MA).

After amplification, reactions were stopped with an equal volume of gel loading buffer (98% deionized formamide, 20 mM EDTA [pH 8.0], 0.025% bromophenol blue, 0.025% xylene cyanol). Immediately before electrophoresis, samples were denatured at 94°C for 5 min and put on ice. AFLP fragments were resolved in 5% polyacrylamide, 8 M urea gels, and visualized by autoradiography. Sizes of AFLP fragments were estimated by comparing to end-labeled molecular weight size standards. Standards used were φX174 RF digested with restriction endonuclease *HaeIII*, λ DNA digested with *HindIII*, and a 30–330 bp AFLP DNA ladder (Life Technologies).

Amplified Fragment-Length Polymorphism Scoring Criteria. AFLP variation was assessed for strain-specific or strain-biased loci. These loci were present exclusively in 1 strain or were contained in the majority of the members of 1 strain but not the other, respectively. Fragments of the same size in 2 unrelated individuals were considered to represent homologous DNA sequences. Each fragment was treated as a dominant allele (character state 1) at a unique AFLP locus. Absence of a fragment in an individual was considered

to represent the presence of an alternative allele (character state 0). Each set of 10 scored loci was referred to as an AFLP fingerprint (Vos et al. 1995). AFLP fingerprints identified in corn strain individuals were designated C1-C9, and rice strain AFLP fingerprints were designated R1-R19.

Initial screening with each *EcoRI* and *MseI* primer pair included at least 4 unrelated individuals of each strain. Upon identification of strain-specific, well-defined loci with high density signals, additional individuals of each strain were examined (up to 37 total per strain).

Data Analysis. Estimates of genetic distance in AFLP polymorphisms within and between strains were calculated from pairwise comparisons of all AFLP fingerprints. Distances were based on the measure 1-M, where $M = N_{ij}/N_T$. M is a matching coefficient, with N_{ij} = the total number of matches (both fragments present or absent) in individuals i and j , and N_T is the total number of fragments scored (RAPDLOT; Kambhampati et al. 1992). Cluster analyses included an unweighted pair group method using arithmetic averaging with bootstrapping (unweighted pair-group method with arithmetic average, 500 replicates, Kambhampati et al. 1992), and a principal coordinate analysis (SYSTAT, Wilkinson 1988).

Amplified fragment-length polymorphism variation within and between fall armyworm strains was quantified using Shannon's information index for phenotypic diversity (King and Schaal 1989). The phenotypic diversity value for a specific strain, H_s , is calculated from $H_s = -\sum p_i \ln p_i$, where p_i is the frequency of a specific AFLP fingerprint in that strain. The proportion of the overall observed polymorphism attributable to within strain variation is H_{avg}/H_T , where H_{avg} is the average of H_s calculated for each

Table 2. Ten AFLP loci selected for fall armyworm strain identification

Primer pair-locus amplified ^a (size of fragment in base pairs)	Frequency of most common allele (1 or 0)	
	Corn ^b	Rice ^b
ECCA/MAGG-1 (250)	1.00 (1)	0.92 (0)
ECCA/MAGG-2 (315)	0.95 (0)	0.75 (1)
ECT/MACT-3 (215)	0.89 (1)	0.80 (0)
ECT/MACT-4 (350)	0.95 (0)	0.72 (1)
ECCG/MAAG-5 (265)	0.97 (1)	0.80 (0)
ECCG/MAAG-6 (125)	0.92 (1)	0.90 (0)
ECCG/MAAG-7 (90)	0.97 (1)	0.75 (0)
ECCG/MACA-8 (320)	0.95 (1)	0.92 (0)
ECCG/MACA-9 (150)	0.81 (1)	0.85 (0)
ECCA/MACG-10 (200)	0.97 (1)	0.80 (0)
<i>n</i>	37	37

^a Primer sequences are given in Table 1.

^b Fall armyworm strain, identified by habitat of origin and mtDNA haplotype.

strain, $H_T = -\sum p \ln p$, is the phenotypic diversity calculated from all groups, and p is the frequency of a specific AFLP fingerprint in all fall armyworm samples. The proportion of polymorphism caused by between strain variation is then $(H_T - H_{avg})/H_T$.

Results

Strong strain biases in the frequencies at many loci indicated that a multilocus approach, employing a combination of AFLP loci, could be used for strain identification (Fig. 1). Ten loci amplified with 5 primer pairs were selected for which the most com-

mon allele in each strain occurred at a frequency of greater than or equal to 70% (Table 2). Twenty-eight different multilocus AFLP fingerprints were identified in the 74 fall armyworms sampled (Table 3). In the rice strain, 19 AFLP fingerprints were identified, and 9 fingerprints were identified in the corn strain. None of the AFLP fingerprints were common to both strains.

Phenetic analyses were conducted to determine if the placement of AFLP fingerprints into groups suggested by the distribution of loci was consistent with our strain designations, and to illustrate the degree of relatedness among the AFLP fingerprints. Both unweighted pair-group method with arithmetic average and principal coordinate analyses indicated 2 main groups of AFLP fingerprints (Figs. 2 and 3, respectively). In both analyses, the same AFLP fingerprints clustered within each group. Group A in each figure contains fingerprints found in 36 corn strain individuals as well as 3 individuals of the rice strain (R1, R17, R18). Group B contains fingerprints found in 34 individuals of the rice strain and in 1 corn strain individual (C3). Although bootstrap values indicated limited support for some nodes, the 4 discordant individuals clearly belong in the cluster groups where they occur (i. e., many nodes support their location in the dendrogram). The overall frequency of individuals with discordant strain assignments based on habitat/mtDNA and AFLP fingerprints was 5% (4/74), 8% in the rice strain (3/37), and 3% in the corn strain (1/37).

The rice strain exhibited greater AFLP variation than the corn strain. A quarter of the rice strain individuals shared a common fingerprint (24%, R9),

Table 3. Presence (1) or absence (0) of fragments in fall armyworm AFLP fingerprints and frequency of each in the corn (C) and the rice (R) strain

Fingerprint	AFLP Loci										Frequency in corn (<i>n</i> = 37)	Frequency in grass (<i>n</i> = 37)
	1	2	3	4	5	6	7	8	9	10		
C1	1	0	1	0	1	1	1	1	1	1	0.62	0.00
C2	1	0	1	0	1	1	1	1	1	0	0.16	0.00
C3	1	1	0	1	0	1	0	0	1	0	0.03	0.00
C4	1	1	1	0	1	0	1	1	1	1	0.03	0.00
C5	1	0	1	1	1	1	1	1	1	1	0.03	0.00
C6	1	0	1	0	1	1	1	0	0	1	0.03	0.00
C7	1	0	1	0	1	0	1	1	1	1	0.03	0.00
C8	1	0	0	0	1	1	1	1	1	1	0.05	0.00
C9	1	0	0	0	1	0	1	1	1	1	0.03	0.00
R1	1	0	1	0	1	0	1	1	0	1	0.00	0.03
R2	0	0	0	0	1	0	0	0	0	0	0.00	0.05
R3	0	0	1	0	0	0	0	0	0	0	0.00	0.03
R4	0	0	1	1	1	1	0	0	0	0	0.00	0.03
R5	0	0	0	0	0	0	1	0	0	1	0.00	0.03
R6	0	0	0	0	0	0	0	0	0	0	0.00	0.05
R7	0	0	1	0	1	0	0	0	1	0	0.00	0.03
R8	0	1	0	1	0	0	0	0	0	1	0.00	0.05
R9	0	1	0	1	0	0	0	0	0	0	0.00	0.24
R10	0	1	0	1	1	1	0	0	1	0	0.00	0.03
R11	0	1	1	1	0	0	0	0	0	0	0.00	0.03
R12	0	1	0	1	0	0	1	0	0	0	0.00	0.14
R13	0	1	0	1	0	0	0	0	1	0	0.00	0.11
R14	1	1	1	1	0	0	0	0	0	1	0.00	0.03
R15	0	1	0	1	0	1	0	0	0	0	0.00	0.03
R16	0	1	0	0	0	0	1	0	0	0	0.00	0.03
R17	0	0	1	0	1	1	1	1	0	1	0.00	0.03
R18	1	1	1	1	1	0	1	1	0	1	0.00	0.03
R19	0	1	0	0	0	0	0	0	0	1	0.00	0.03

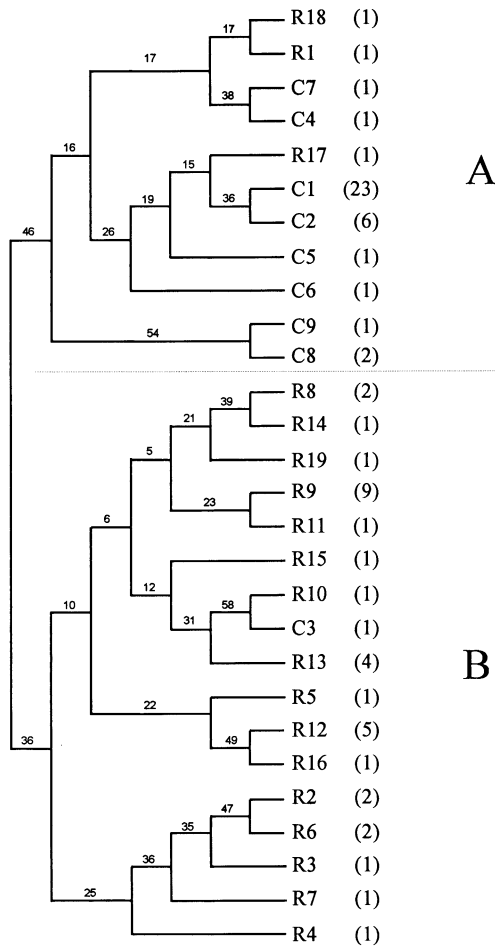


Fig. 2. Dendrogram of 28 fall armyworm AFLP fingerprints resulting from unweighted pair-group method with arithmetic mean cluster analysis. Fingerprints C1-C9 were found in individuals collected in corn with corn strain mtDNA haplotype. Fingerprints R1-R19 were found in individuals collected in pasture grasses with rice strain mtDNA haplotype. Bootstrap support (percent of 500 replicates) is indicated for each branch. Numbers in parentheses to the right indicate the number of individuals that had each AFLP fingerprint.

whereas more than half of the individuals in the corn strain possessed a common AFLP fingerprint (62%, C1). Within-strain phenotypic diversity estimated for rice, 2.7, was twice the value estimated for corn, 1.3. AFLP variation was greater within fall armyworm strains than between strains. The proportion of overall diversity attributable to within strain polymorphism was 76%, whereas the contribution of among strain polymorphism to the overall diversity was 24%. Mean frequencies of the most common allele in each strain (corn strain, $0.94 \pm SE 0.05$; rice strain, $0.82 \pm SE 0.07$) were consistent with greater variation in the rice strain but were not significantly different.

Discussion

To test the usefulness of AFLP variation for strain identification, we intentionally selected individuals to represent each strain unambiguously based on habitat and mtDNA genotype. Typically, studies of hybridization among strains or species where a hybrid zone exists initiate marker surveys using allopatric populations that have “pure” species characteristics. There are no such known populations of fall armyworm, thus an alternative approach to construct pure strains was required.

Analysis of AFLP variation in fall armyworm revealed 2 assemblages. These groupings were consistent with our strain assignments in 95% of the samples. Within our corn strain assignments, 97% of the individuals clustered together in 1 assemblage, and within our rice strain designates, 92% clustered together. Four individuals (5%) contained an AFLP genotype inconsistent with our strain assignment based on host and mtDNA.

Similar inconsistencies have been obtained with other markers pursued to distinguish the 2 strains. To date, no pair of markers shows complete congruence with each other or host of origin. In other words, allozyme or mtDNA genotypes characterizing 1 strain can occur in individuals collected on the other strain’s host. Individuals on a single host can contain an allozyme genotype characteristic of 1 strain but a mtDNA genotype of the other. In a survey of 584 samples, 10% of the individuals exhibited discordance between allozymes and mtDNA (Prowell 1998). In the current, smaller scale study, AFLPs exhibited somewhat better congruence (5%) with mtDNA.

There is a consistent bias with respect to the habitat where discordances occur. Samples collected in the corn habitat exhibit more genetic inconsistencies between allozymes and mtDNA. For example, contradictory allozyme and mtDNA genotypes occurred in 16% of corn field samples and only in 3% of the rice and pasture habitat samples (Prowell 1998). In addition, 18% of the individuals collected in corn were likely to be members of the rice strain based on agreement between allozyme and mtDNA genotypes, whereas only 2% of the individuals collected in pastures or rice fields were likely to be members of the corn strain (Pashley 1989). This suggests that the rice strain more readily uses corn as a host than the corn strain uses rice and forage grasses. Our AFLP results are consistent with other genetic markers in this habitat-related bias. More rice strain AFLP fingerprints were found in corn than the reverse.

Explanations for conflicts between genetic markers may have an historical basis or may be linked to interstrain hybridization. Shared alleles or genotypes in reproductively isolated strains could result from polymorphic traits in the common ancestor to the 2 strains that did not become fixed during subsequent divergence leading to the 2 strains. Alternatively, if the ancestors to the 2 strains evolved or possessed fixed differences, shared genotypes could result from erosion of differences by gene flow through hybridiza-

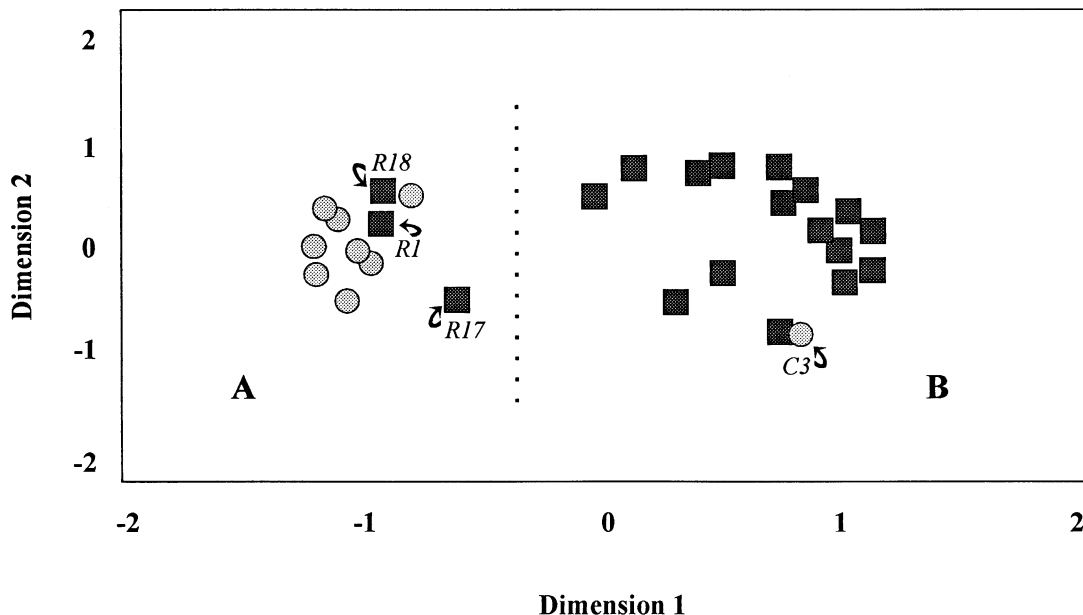


Fig. 3. Principal coordinate analysis of 28 fall armyworm AFLP fingerprints. Circles represent patterns found in individuals collected in corn with corn strain mtDNA haplotypes. Squares represent patterns found in individuals collected in pasture grasses with the rice strain mtDNA haplotype. AFLP fingerprints and habitat/mtDNA were discordant in the individuals indicated with an arrow (R1, R17, R18, C3).

tion. Given that we have multiple genetic markers that show strong bimodal distributions associated with host plant use, we are now in a position to try to distinguish between these 2 hypotheses. Future studies will encompass full genotypic screening of a broader temporal and geographic sample of fall armyworm populations to address this issue.

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