that methionine was retained. A sedimentation equilibrium experiment on a 0.1 mM protein solution revealed a molecular weight of 11,800, which indicates that most of the protein molecules exist as monomers. Size exclusion chromatography also showed a monomeric molecular size. Nuclear magnetic resonance measurements of 2 mM solutions of the protein [in 30 mM KCl, 20 mM phosphate buffer, and 1 mM dithiothreitol (pH 6.0) at 30°C] were performed with Bruker AMX500 and DMX500 spectrometers. Most of the observable ^{1}H, ^{15}N, and ^{13}C nuclei were given resonance assignments by means of the following 3D experiments: ^{15}N-edited total correlation spectroscopy (TOCSY)–HSQC, CBCA(CO)NH, CBCANH, HBHA(CBCACO)NH, HBHA(CBCA)NH, HNCO, and HN(CA)CO for the backbone nuclei [A. Bax and S. Grzesiek, in *NMR of Proteins*, G. M. Clore and A. M. Gronenborn, Eds. (Macmillan, London, 1993), pp. 33–52], and HEHOHEHAHA [A. Majumdar, H. Wang, R. C. Morshauser, E. R. P. Zuiderweg, *J. Biomol. NMR* **3**, 387 (1993)] for the side chain nuclei. Distance information was collected using two-dimensional homonuclear nuclear Overhauser effect spectroscopy (NOESY) with a 100-ms mixing time, and 3D (^{1}H,^{13}C) and (^{1}H,^{15}N) NOESY-HSQC spectra with a 100-ms mixing time. For torsion angle constraints, the backbone vicinal coupling constants ($^{3}J_{HN,H\alpha}$) were estimated from a two-dimensional heteronuclear multiple quantum coherence J (HMQC-J) spectrum [L. E. Kay and A. Bax, *J. Magn. Reson.* **86**, 110 (1990)] of uniformly ^{15}N-labeled αCTD. The NOE connectivities from strong, medium, and weak cross-peaks were assumed to correspond to the upper limits for proton-proton distances of 3.0, 4.0, and 5.0 Å, respectively. Because stereospecific assignments for the methyl and methylene protons have not yet been performed, appropriate corrections were added for constraints including the pseudoatoms. The hydrogen bond constraints were added for slowly exchanging amides as upper and lower limits of 3.0 and 2.7 Å for N–O, respectively, and 2.0 and 1.8 Å for H–O, respectively. For torsion angle constraints, residues that gave $^{3}J_{HN,H\alpha}$ values of <5.5 Hz, 8.0 to 10 Hz, and >10 Hz were estimated to have torsion angles φ of −90° to −40°, −160° to −80°, and −140° to −100°, respectively, except for the cases of very strong intraresidue HN-Hα NOEs. In total, 822 NOE constraints, 52 dihedral angle constraints, and 19 sets of hydrogen bond constraints were used. Simulated annealing calculations [M. Nilges, G. M. Clore, A. M. Gronenborn, *FEBS Lett.* **229**, 317 (1988)] were performed on Silicon Graphics workstations with X-PLOR [A. T. Brünger, version 3.1 (Yale Univ. Press, New Haven, CT, 1992)]. Three hundred structures were calculated, and 50 structures with distance violations of <0.3 Å and angle violations of <5° were obtained.

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11. A DNA titration experiment was done by adding 0.05, 0.1, 1.0, and 1.2 molar equivalents of DNA to the protein at 30 mM KCl. However, signal broadening was so severe that most peaks were barely observed. We extended the experiment by increasing the KCl concentration to 800 mM in the presence of 0.1 molar equivalent of DNA. Almost every peak reappeared at the 800 mM salt condition. No precipitation occurred throughout these experiments. The solution condition of 500 mM KCl for Fig. 2A was chosen as the best for observation of strongly perturbed peaks separately from others. To determine whether the signal losses were specifically caused by the UP-element DNA, we performed a similar experiment in the presence of a 22-bp DNA duplex [d(TAATGTGAGTTAACTCACATTA)] that corresponded to the consensus binding sequence of CRP. We observed only slight shifts of peak positions for two or three residues, but we could not see any disappearance of peaks because of broadening. Thus, the drastic change in the spectrum in the presence of the UP element was clearly distinguished from the control experiment.
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A Population Genetic Test of Selection at the Molecular Level

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The role of natural selection in molecular evolution has been inferred primarily by rejection of null hypotheses based on neutral theory, rather than by acceptance of specific predictions based on selection. In this report, a population genetic test of a specific prediction for selection on DNA polymorphism is presented. Pyrethroid insecticide use constitutes an experiment for which form of selection and molecular target (voltage-gated sodium channels) are both known. As predicted, differential pyrethroid selection on tobacco budworm populations generated significant geographic heterogeneity in sodium channel marker allele frequencies, compared with arbitrary loci.

Many studies have sought to test the null hypothesis of neutral evolution of DNA sequence variation in populations. However, the lack of specific prior predictions based on selection has weakened such tests. Even for the most well-studied case of the high-to-low latitudinal clines in the *Adh* fast-slow polymorphism in *Drosophila melanogaster*, the form of selection presumed to maintain this polymorphism remains elusive, although patterns of polymorphism are consistent with a model of balancing selection (*1*).

If a specific form of selection is expected at a particular locus, then a prediction may be developed on the basis of selection rather than neutrality, which may be tested with the use of appropriate statistics. Differentiation in allele frequencies among populations can be estimated by F_{st}, the between population component of standardized genetic variance. Population structure and historical contingencies (range expansions or contractions) have genome-wide effects on gene frequency variation between populations, whereas selection affects variation only at target loci. Differentiation in the form of clines of allele frequency for some allozyme loci, and not for others, has been taken as evidence that spatially variable selection has been acting at such loci, whereas gene flow has homogenized frequencies at other unselected loci (*2*). However, clinal patterns for some loci may result from historical patterns of colonization and subsequent admixture, whereas nonclinal loci are homogenized by selection, acting uniformly in space and causing convergence of frequencies over a wide area (*3*). The study of nucleotide variation has permitted some resolution of this dilemma for the *Adh* cline in *D. melanogaster* (*4*). Such studies highlight the difficulties of testing for selection at the molecular level in natural populations, in the absence of prior expectations about the strength, duration, or loci of action of selection.

The widespread use of insecticides in agriculture has resulted in the rapid evolution of resistance for many pest species. Such natural "experiments" can enable explicit prediction of the outcome of selection at the molecular level. Pyrethroid insecticides act on voltage-gated sodium channels in nerve membranes (*5*). Resistance to the pyrethroid permethrin in a field-derived strain of the tobacco budworm (*Heliothis virescens*), a major cotton pest, is linked to a DNA marker for a sodium channel locus *hscp*, homologous to the *para* locus of *D. melanogaster* (*6*). Levels of resistance are known to vary considerably among populations, and resistance has arisen in response to selection in just the last 10 years (*7*). Population genetic surveys in North America, ranging from Texas to Georgia for 13 allozyme loci (*8*), and from Sonora, Mexico, to Georgia for mitochondrial DNA markers (*9*), have shown that tobacco budworm populations are little differentiated, which

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is consistent with high rates of gene flow across large distances.

We predicted that tobacco budworm populations would be highly differentiated at the target sodium channel locus as a result of geographically varying levels of selection by pyrethroids, while remaining essentially undifferentiated at other arbitrarily chosen loci as a result of gene flow. To test this hypothesis, we collected 660 adult male tobacco budworms in five samples from four widely separated U.S. locations [Georgia, Texas, Arizona, and Louisiana (two different times)]. Individuals were tested for a phenotype of "knockdown" at one of two doses of the pyrethroid cypermethrin (*10*). Resistance to the paralytic knockdown effect of these insecticides, rather than survival, was the phenotype definition chosen for comparison to other field studies (*7*).

We then examined variation in a small DNA marker *Hpy* for the sodium channel locus *hscp* (*11*) and another similar sized marker *Hejs* in the juvenile hormone (JH) esterase locus, which is unlinked to *hscp* and has no known role in pyrethroid resistance (*12*). Variation was scored by denaturing gradient gel electrophoresis that, under suitable conditions, can detect nearly all nucleotide polymorphisms in a region of interest (*13*). We assumed that the uniformly low F_{st} estimates observed for allozyme loci (ranging from near zero to 0.005) at all scales of spatial sampling reliably reflect high gene flow between tobacco budworm populations (*8*). There is no evidence that any of these allozyme loci are involved in insecticide resistance in insect species. Thus, we could make a specific prediction for the relative magnitudes of the F_{st} estimates for the marker loci in this study.

The value of F_{st} for the arbitrary *Hejs* marker was predicted to be small and not significantly different from the allozyme F_{st} estimates. The F_{st} for the sodium channel *Hpy* marker was predicted to be significantly greater than either allozyme or *Hejs* F_{st} estimates as a result of close genetic linkage to one or more unknown mutations in the sodium channel that had been favored by recent, intense pyrethroid selection in some populations. We decided, in advance, to accept the hypothesis that selection had been acting at the sodium channel locus only for this outcome of the study. Outcomes in which F_{st} for *Hpy* was indistinguishable from that for *Hejs*, or in which F_{st} for *Hejs* was significantly greater than allozyme F_{st} estimates, both entailed rejection of this hypothesis.

The five samples varied significantly in cypermethrin knockdown, showing that pyrethroid selection had been geographically variable, resulting in heterogeneous levels of resistance (Fig. 1A). It would be desirable to be able to reconstruct treatment histories to give some estimate of selection intensity in each of the regions sampled, as has been done for more restricted populations (*14*). Although the local abundance and rate of insecticide treatment for both bollworms and budworms on cotton had been similar in Georgia and Louisiana before this collection, the incidence of pyrethroid resistance was consistently much lower in Georgia (*15*). This may be attributed to a much larger untreated reservoir population on alternative wild host plants in the southeastern United States, and thus a much lower net selection intensity averaged over the local population (*16*).

A total of 50 alleles for *Hpy* and 41 alleles for *Hejs* were distinguishable on the basis of gel-mobility differences. The *Hpy* allelic diversities of samples were significantly correlated with knockdown at pesticide concentrations of 5 μg (Fig. 1B). However, no such correlation was observed for *Hejs* allelic diversities (Fig. 1B). This pattern is consistent with a process of selective removal of uncommon *Hpy* alleles in the more heavily selected populations, especially in Louisiana.

Analysis of the F_{st} statistics for the two marker loci confirmed our prediction exact-

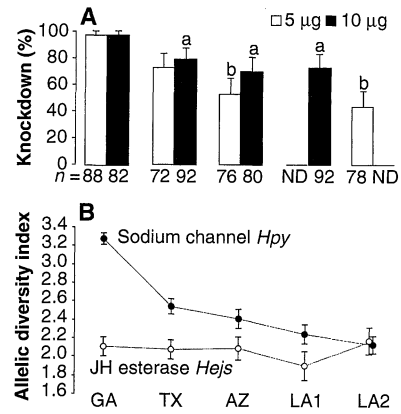

Fig. 1. (**A**) Proportion of adult male tobacco budworms dead or knocked-down ("knockdown"), with 95% confidence intervals, in bioassays with 5 or 10 μg of cypermethrin per vial for each sample (*10*). The samples, ordered by *Hpy* allelic diversities, were as follows: GA, Sparks, Georgia, August 1990; LA1, Winnsboro, Louisiana, August 1990, and LA2, same location, October 1990; TX, College Station, Texas, September 1990; AZ, Yuma, Arizona, October 1990. The numbers below the bars are sample sizes; ND, no data. Within each dose, knockdown proportions for samples with the same letters were not significantly different at the 5% level in χ^2 tests. (**B**) Means and standard errors of *H*, allelic diversity indices, for sodium channel *Hpy* and JH esterase *Hejs* markers, for each sample (*20*). Means and standard errors were estimated by the jackknife over individuals (*21*). Kendall's rank correlation coefficient between *Hpy* diversity and knockdown with 5 μg of pesticide was significant at the 5% level. Correlations between *Hpy* diversity and knockdown with 10 μg of pesticide, and between *Hejs* diversity and knockdown in either bioassay, were not significant at the 5% level.

Table 1. Differentiation among samples as measured by F_{st} and allelic diversities *H* for sodium channel *Hpy* and JH esterase *Hejs* marker loci and for allozyme loci. Values for F_{st} were calculated with the sample size correction of Nei and Chesser (*19*). Mean and standard errors were estimated by the jackknife over individuals (*21*). Hypothesis tests were performed by deriving a null sampling distribution for F_{st} by repeatedly (1000 times) reallocating genes randomly among all individuals and recalculating F_{st}. The "tail" proportion of such resampled null F_{st} estimates that equaled or exceeded the observed value of F_{st} is an estimate of the probability (Pr.) of obtaining the observed value of F_{st} under the null hypothesis ($F_{st} = 0$). For values of *H*, see Fig. 1B.

Locus	$F_{st} \pm$ SE	Pr. ($F_{st} = 0$)	$H \pm$ SE
Sodium channel *Hpy* marker, 50 alleles	0.041 ± 0.005	<0.001	2.86 ± 0.04
Hpy, pooling alleles 1 to 4	0.031 ± 0.006	<0.001	2.07 ± 0.06
JH esterase *Hejs* marker, 41 alleles	0.001 ± 0.003	0.16	2.09 ± 0.06
Thirteen allozyme loci*	0.002 ± 0.001	<0.0001	

*Date are from a larger collection at 60 pheromone trap locations between Georgia and east Texas in 1989 (*8*). Only total F_{st} is shown. The F_{st} for between traps, within sites, and within regions had the largest contribution to total F_{st}. Between regions F_{st} was very low. Mean and standard errors were estimated by the jackknife over loci. Allelic diversity is not shown, as no comparison was needed.

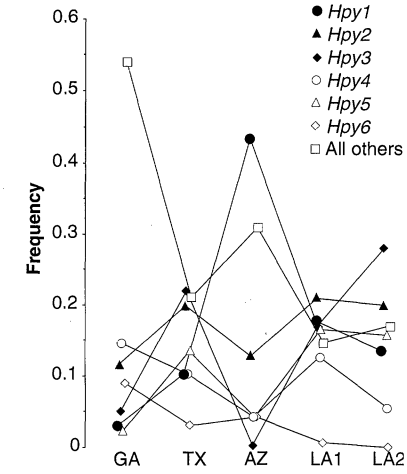

Fig. 2. Frequencies of sodium channel *Hpy* marker alleles 1 through 6, and all other alleles pooled, in each sample (each of the alleles that went into the pool was less than 5% of the total sample: 43 for GA, 33 for TX, 34 for AZ, 19 for LA1, and 15 for LA2).

ly. The value of F_{st} estimated for the sodium channel *Hpy* marker was an order of magnitude greater than for that of *Hejs* or the allozyme loci (Table 1). This was evident in the heterogeneity among samples in *Hpy* allele frequencies (Fig. 2). The *Hpy1* allele, which previously had been linked to permethrin resistance in a field-derived strain (6), was the most common in the entire collection and was generally more frequent in the more resistant samples (Fig. 2). Some *Hpy* marker alleles are expected to "hitchhike" along with linked resistance-conferring mutations in or near the sodium channel locus. However, this linkage disequilibrium is continually broken down by recombination, and no single *Hpy* allele is necessarily expected to be uniquely associated with resistant phenotypes.

Also as predicted, the estimated value of F_{st} for the arbitrary marker *Hejs* was low and not significantly different from zero or from the low F_{st} estimates for the allozyme loci (Table 1). *Hejs* allele frequencies were markedly homogeneous across samples, which is consistent with the low value of F_{st} (Fig. 3). It is possible that *Hejs* was in fact as heterogeneous among samples as was *Hpy* but that this was undetectable because of the skewed allelic distribution of *Hejs*, with one very common allele (Fig. 3). To test this possibility, we pooled the four most common *Hpy* alleles to construct an allelic distribution for *Hpy* with an allelic diversity index similar to that of *Hejs* for the entire collection (Table 1). The pooled F_{st} estimate for *Hpy* was only slightly different, which indicates that the difference in F_{st} estimates was not an artifact of different allelic distributions nor attributable to a single allele (Table 1). The F_{st} estimate for the *Hpy* marker was therefore quite robust.

Any contrast among loci in estimates of F_{st}, as a test of the null hypothesis of neutral molecular evolution, is weak because many outcomes are as consistent with neutral theory as with any number of unspecified models of selection (*17*). In this case, however, only one outcome was predicted for pyrethroid selection, and this therefore formed a null hypothesis of selection. The expectation from neutral theory, in the absence of evident population subdivision, was that F_{st} estimates for any allozyme and DNA markers should all be small and statistically indistinguishable. Outcomes in which one or more loci have unusually large values of F_{st} could arise from stochastic neutral processes, but should be uncommon (*17*). A neutral outcome in which precisely the locus of interest, and no other locus, has a large value of F_{st} must, correspondingly, be rare.

Here, we have attempted to bring together two scientific programs that historically have been isolated: the study of molecular evolution and the study of insecticide resistance in pest populations. In our test for selection at the molecular level, the specific outcome of selection could be predicted for a particular locus and then tested. Despite much evidence for the involvement of particular mutations in insecticide resistance and for persistence or spread of these mutations in populations (*18*), we have shown that molecular mutations at the known target locus are under strong, recent selection in field populations.

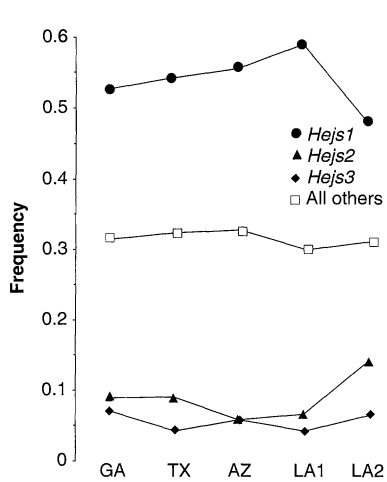

Fig. 3. Frequencies of JH esterase *Hejs* marker alleles 1 to 3, and all other alleles pooled, in each sample (each of the alleles that went into the pool was less than 5% of the total sample: 30 for GA, 31 for TX, 32 for AZ, 23 for LA1, and 24 for LA2).

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10. Inner surfaces of 20-ml glass scintillation vials were coated with either 5 μg or 10 μg of cypermethrin in acetone and allowed to dry by rolling. Adult male tobacco budworms were captured in pheromone bait traps and placed individually in vials and held at ~25°C. After 24 hours, they were scored as dead or knocked down, or alive and active, and all were frozen at −80°C for genotype analysis. Knockdown was defined as the inability to turn upright after being turned upside-down.
11. Genomic DNA was prepared by homogenizing moths in 750 μl of grinding buffer [0.1 M tris-HCl (pH 8.0), 0.1 M NaCl, 50 mM EDTA, 6.8% w/v sucrose, and 0.5% w/v SDS]. The aqueous phase was extracted three times with 750 μl of phenol (pH 8) and once with 750 μl of chloroform. Nucleic acids were recovered after a 30-min incubation with 1 M potassium acetate and 50% isopropanol by centrifugation at 12,000*g* for 15 min. Pellets were washed with 70% ethanol, dried, and resuspended in 20 μl of TE [10 mM tris-HCl (pH 8) and 100 μM EDTA]. Polymerase chain reactions consisted of 0.1 to 0.5 μg of genomic DNA, 30 mM tricine-HCl (pH 8.4), 5 mM β-mercaptoethanol, 0.01% gelatin, 0.2 mM for each of the four deoxynucleotide triphosphates, 4.5 mM $MgCl_2$, 0.4 U of Taq DNA polymerase, 0.2 μM primer *CL/Hp4399-* (5′-GCCCGCCGCCGCGCCCCGCC-GCCACCGCGCCGCCCGCCCCCGCGTCGTTCA-TGATCTGTATCCA), and 0.2 μM primer *Hp4211+* (5′-CTGATCTTCGCCATCATGGG), with a final volume of 20 μl. Reactions were incubated for 20 cycles of 94°C for 35 s, 56°C for 1 min 30 s, and 72°C for 30 s; then for 14 cycles of 94°C for 35 s, 56°C for 1 min 30 s, and 72°C for 1 min; and finally for one cycle of 94°C for 35 s, 56°C for 15 min, and 72°C for 1 min. Reactions were precipitated, resuspended in 5 μl of dye-loading buffer, and separated on a vertical polyacrylamide gel (25 cm by 15 cm by 0.5 mm) ranging from 35 to 45% denaturant concentration, at 73 V for 14 hours at 60°C. DNA was detected by ultraviolet fluorescence after a 15-min staining in ethidium bromide (0.5 μg/ml). Gel, buffer, and denaturant solutions were as described [R. M. Myers, T. Maniatis, L. S. Lerman, *Methods Enzymol.* **155**, 501 (1987)]. Primers were based on the published sequence (*6*).
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$$H = -\sum_{i=1}^{k} p_i \log(p_i)$$

where p_i is the frequency of allele $i = 1$ to k.
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