

3-2012

Genetic Differentiation of Western Corn Rootworm Populations (Coleoptera: Chrysomelidae) Relative to Insecticide Resistance

Hong Chen

University of Nebraska-Lincoln, entomail@gmail.com

Haichuan Wang

University of Nebraska-Lincoln

Blair D. Siegfried

University of Nebraska-Lincoln, bsiegfried1@unl.edu

Follow this and additional works at: <http://digitalcommons.unl.edu/entomologyfacpub>



Part of the [Agriculture Commons](#), [Agronomy and Crop Sciences Commons](#), and the [Entomology Commons](#)

Chen, Hong; Wang, Haichuan; and Siegfried, Blair D., "Genetic Differentiation of Western Corn Rootworm Populations (Coleoptera: Chrysomelidae) Relative to Insecticide Resistance" (2012). *Faculty Publications: Department of Entomology*. 527.

<http://digitalcommons.unl.edu/entomologyfacpub/527>

This Article is brought to you for free and open access by the Entomology, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Faculty Publications: Department of Entomology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Published in *Annals of the Entomological Society of America* 105:2 (2012), pp. 232–240;

doi: 10.1603/AN11124

Copyright © 2012 Entomological Society of America; published by Oxford University Press. Used by permission.

Submitted July 13, 2011; accepted December 8, 2011.

Genetic Differentiation of Western Corn Rootworm Populations (Coleoptera: Chrysomelidae) Relative to Insecticide Resistance

Hong Chen, Haichuan Wang, and Blair D. Siegfried

Department of Entomology, University of Nebraska–Lincoln, Lincoln, Nebraska

Corresponding author – Blair D. Siegfried, email bsiegfried1@unl.edu

Abstract

As the single most important pest of field corn, *Zea mays* L., throughout most of the Corn Belt, the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), has undergone repeated selection for resistance to a variety of insecticides that persist widely among Nebraska populations. In this study, we used 11 microsatellite markers to genotype two populations with high levels of resistance to methyl-parathion and aldrin (Polk and Stromsburg), two populations with low and intermediate levels of resistance (Mead and Clay Center) from Nebraska, and one population from outside the Corn Belt (Safford, Arizona). The genetic diversity measured by observed heterozygosity (H_o) was reduced 15–32% in the highly resistant populations compared with the more susceptible populations in Nebraska. Significant genetic differentiation was detected between the resistant and susceptible populations (Polk and Stromsburg versus Mead and Clay Center) in Nebraska ($F_{ST} = 0.016$) and between all the populations from Nebraska and Arizona ($F_{ST} = 0.059$). The average observed heterozygosities in the populations were positively correlated with insecticide susceptibility based on mortality at diagnostic concentrations of aldrin and methyl-parathion, respectively. These results indicate that the insecticide selection from exposure to aldrin and methyl-parathion may be a contributing factor in shaping the genetic structure of western corn rootworm populations in Nebraska. Factors including isolation by distance and a *Wolbachia*-induced breeding barrier may have contributed to differentiation of rootworm populations from Nebraska and Arizona.

Keywords: *Diabrotica virgifera virgifera*, population structure, insecticide selection, aldrin, methyl-parathion

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is arguably the single most important pest of field corn, *Zea mays* L., throughout most of the Corn Belt both in terms of crop losses and the use of synthetic insecticides. *D. v. virgifera* has been suggested to have originated from Central America, where it is believed to have used corn as a larval host for $\approx 5,000$ yr based on the range of native corn with resistance to rootworms (Melhus et al. 1954, Krysan and Smith 1987). Before 1930, the insect's distribution was limited to the adjacent areas of Colorado, Kansas, and Nebraska, where it became a pest of continuous corn production. Root injury was reported in southwestern Nebraska in 1929 and 1930 (Tate and Bare 1946). From 1930 to 1955, the rootworm expanded eastward at an average of 20 km/yr and by the mid-1940s, severe damage to corn was found in central Nebraska. Over the past 50 yr, the rate of eastward expansion increased to an average of 40 km/yr, and the current distribution of *D. v. virgifera* extends to the East Coast (Meinke et al. 2009). The first established population of western corn rootworm in Europe was detected near Belgrade, Serbia, in 1992 which has expanded to include parts of 11 countries, extending from Austria to the Ukraine and from Southern Poland to Northern Bulgaria (Ciosi et al. 2008a,b).

Cyclodiene insecticides were widely used as soil insecticides for rootworm management throughout the 1950s and early 1960s. By 1959, almost 1,000 metric tons of aldrin had been applied as a soil insecticide in Nebraska alone (Ball 1983). Failures to control rootworms with these compounds were first noted in Nebraska in 1959 (Roselle et al. 1959), and further evaluations revealed the magnitude and rapid development of resistance (Roselle et al. 1960, 1961). Differences in susceptibility as high as 1,000-fold were detected among field populations in Nebraska and provided the first direct evidence for the evolution of resistance (Ball and Weekman 1962, 1963). The development of cyclodiene resistance coincided with the rapid eastward range expansion of this pest. All tested populations east of the Missouri River in the United States have been and continue to be highly resistant to cyclodiene insecticides (Siegfried and Mullin 1989; Parimi et al. 2006; B.D.S., unpublished data). Furthermore, a recent investigation of cyclodiene susceptibility among invasive European populations has revealed that among nine field populations examined, all were highly resistant to aldrin (Ciosi et al. 2008a).

An alternative strategy for chemical control of western corn rootworm involves aerial application of insecticides (carbamates and organophosphates) that mainly targets adult females to reduce oviposition and economic damage by the subsequent generation (Pruess et al. 1974). This practice became widely adopted in certain areas of the western Corn Belt (Meinke et al. 1998), and in some areas of Nebraska, aerially applied Penncap-M (methyl-parathion) was used almost exclusively (Meinke et al. 1997) over relatively large areas and in consecutive years. Control failures of aerially applied methyl-parathion were first reported in the early 1990s, and resistance was documented in rootworm adults from several Nebraska populations (Meinke et al. 1998, Zhou et al. 2002, Parimi et al. 2006). The distri-

bution of resistant rootworms was initially restricted to areas of the state where adult management had been practiced for >10 yr, whereas in areas relying on soil insecticides and crop rotation, the beetles remained susceptible.

High levels of resistance to these insecticides have persisted among rootworm populations in Nebraska, although selective pressures have changed dramatically as a result of changing management strategies. Cyclodiene insecticides were banned from use in the United States in 1972 because of their extreme persistence and widespread environmental contamination. Adult management with microencapsulated methyl-parathion was largely replaced by crop rotation and soil insecticides in the mid-1990s (Parimi et al. 2006) and more recently transgenic *Bacillus thuringiensis* (Bt) maize for rootworm control has become widely adopted (Gray et al. 2009). The traits of resistance to both aldrin and methyl-parathion remain relatively stable although considerable variation in susceptibility among populations over relatively short distances (<50 km) has been detected in Nebraska (Ball and Weekman 1963; Meinke et al. 1998; Parimi et al. 2006; B.D.S., unpublished data). The well-documented history of the development and spread of resistance to the two different insecticide classes in this species may provide important information related to the movement of resistance conferring genes as well as population genetic structure.

Population genetic structure plays a central role in the evolution of insecticide resistance (McKenzie 1996, Hartl and Clark 2007) and has become an essential component of resistance management strategies for transgenic Bt crops. Various genetic markers have been developed and used to study genetic diversity, phylogeny, and population genetics of the western corn rootworm (Krysan et al. 1989, Simon et al. 1994, Szalanski and Powers 1996, Szalanski et al. 2000, Clark et al. 2001a, Eben and Espinosa de los Monteros 2004, Gillespie et al. 2004, Kim and Sappington 2005b, Miller et al. 2007, Kim et al. 2007, Kim et al. 2008a, Coates et al. 2009). Low levels of genetic differentiation among rootworm populations have been reported for populations from west Texas to the northeastern United States (Szalanski et al. 1999, Kim and Sappington 2005a, Miller et al. 2006). However, Kim and Sappington (2005a) found that populations from Dodge City, Kansas, and Champaign, Illinois, were somewhat isolated from the others. In a later study on genetic variation associated with crop-rotation tolerance, Miller et al. (2006) used both microsatellite and amplified fragment-length polymorphism markers and detected low but significant genetic differentiation between the rotation tolerant populations from Illinois and wild-type populations from Iowa. Multiple invasive events across the Atlantic Ocean and into Europe by the rootworm also have been inferred from microsatellite markers (Miller et al. 2005, Ciosi et al. 2008b) where at least three direct introductions from North America into Europe and two intra-European introductions were identified.

Western corn rootworm populations throughout the Corn Belt are infected with a *Rickettsia*-like bacterium in the genus of *Wolbachia* (Degrugillier et al. 1991, Giordano et al. 1997, Clark et al. 2001b). These intracellular bacteria are grouped together phylogenetically (Division A; Giordano et al. 1997, Clark et al. 2001b, Segura-Leon 2004) and induce unidirectional cytoplasmic incompatibility between infected males and uninfected females (Giordano et al. 1997). It has been suggested that *Wolbachia*-induced cytoplasmic incompatibility may have the potential to drive beneficial genes within pest populations (Sinkins and O'Neill 2000).

In this study, we used 11 microsatellite markers to reveal genetic differentiation of rootworm populations that exhibit significant variation in resistance to aldrin and methyl-parathion. We hypothesize that variable selection pressures, as inferred from insecticide resistance status, shape the genetic structure of rootworm populations. We report estimates of genetic diversity and differentiation between populations with varying levels of insecticide susceptibility that are separated by different geographic distances. In addition, the *Wolbachia* infection status for each population was assessed to evaluate its possible role in genetic differentiation.

Materials and Methods

Sample Collection

Adult western corn rootworms were collected from corn plants at four sites in southeastern Nebraska and one site in southeastern Arizona (Fig. 1). Population samples (Polk and Stormsburg) from York County were selected to represent major corn production regions in Nebraska where cyclodiene and organophosphate insecticides have been used extensively and where resistant populations are well documented (Ball and Weekman 1963, Meinke et al. 1998). Susceptible populations (Clay Center and Mead) were previously identified from University of Nebraska research and extension farms, based on survival at diagnostic level insecticide bioassays for both aldrin and methyl-parathion (B.D.S., unpublished data). The population from Safford, Arizona, represents a population that is separated from the major U.S. corn-growing areas and is in proximity to the proposed ancestral origin of the species in Central America (Krysan and Smith 1987).



Figure 1. Sampling sites of western corn rootworm beetles in Nebraska and Arizona.

DNA was extracted individually from fresh beetles or freshly frozen beetles stored at -80°C , by sodium dodecyl sulfate extraction methods (Chen et al. 2010). For insecticide bioassays, field-collected beetles were maintained on fresh sweet corn ears under ambient conditions of light and humidity for 2–3 d before bioassays.

Microsatellite Genotyping

For each population, 50 beetles were individually genotyped at 11 microsatellite loci, including DVV-D2, DVV-D4, DVV-D5, DVV-D8, DVV-D9, DVV-D10, DVV-D11, DVV-T2, DVV-T3, DbA05, and DbA07 (Kim and Sappington 2005b, Waits and Stolz 2008, Kim et al. 2008b), and genotyping was conducted using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, California). Master mixes from Thermo Fisher Scientific (1.1X, Epsom, Surrey, UK) or Phusion (Finnzymes OY, Keilaranta, Espoo, Finland) were used for polymerase chain reaction (PCR) amplifications of the microsatellites. For each locus, up to three PCR reactions were attempted, and if still unsuccessful, the locus was recorded as unknown.

Microsatellite Data Analyses

Raw data from the CEQ were checked for accuracy with the program MICRO-CHECKER (Oosterhout et al. 2004). Microsatellite polymorphism (number of alleles and heterozygosity per locus), inbreeding coefficients (F_{is}), Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were calculated or tested using GENEPOP (Raymond and Rousset 1995). Conformance with HWE was tested for each locus and population, and the Bonferroni correction was applied for multiple comparisons. The F_{is} statistics and probability test were used to determine whether distortion from HWE resulted from deficient or excessive heterozygosity. Because the probability test is robust to low allele frequencies, rare alleles were not pooled. Variation in heterozygosity among populations was tested for significance using analysis of variance (ANOVA) with population, individual, locus, interactions of loci, and individuals as factors (Weir 1990). All factors except locus were treated as random effects. Likelihood and probability tests were performed to detect genotypic linkage disequilibrium for pairs of loci in each population (Raymond and Rousset 1995).

To identify genetic structure among the populations, F-statistics (F_{ST}) of overall, pairwise, and between-population groups (Nebraska versus Arizona, Polk and Stromsburg [high resistance frequencies] versus Clay Center and Mead [low resistance frequencies]) and isolation-by-distance effects were estimated and tested using FSTAT (Goudet 1995) or GENEPOP (Raymond and Rousset 1995). The standard deviations of F_{ST} were obtained for each locus by a jackknife procedure over all the alleles, and these deviations were used to test for statistical significance. Genetic differentiation between population groups was analyzed by ANOVA. The isolation-by-distance model was tested by linear regression of pairwise $F_{ST}/(1 - F_{ST})$ values against the natural logarithm of straight-line geographic distance between pairs of sampling sites (Rousset 1997). Statistical significance of the regression was tested using the Mantel test with 2000 permutations. The pairwise geographic distance ranged from 19 to 1,490 km. A dendrogram with bootstrap confidence values was created based on the pairwise genetic distances using the unweighted pair group method with arithmetic average in TFGA (Miller 1997).

Insecticide Bioassays

The bioassays with diagnostic concentrations of aldrin (Ciosi et al. 2008a) and methylparathion (Zhou et al. 2002), based on lethal concentration (LC)₉₉ for a susceptible laboratory strain, were conducted in 20-ml insecticide-coated glass scintillation vials (VWR, West Chester, Pennsylvania), with 10 beetles per vial. Bioassays were replicated 10 times for each population. Significance of Pearson correlation between population mortalities and observed heterozygosities was tested for the two chemicals by using MINITAB 15 (Minitab, Inc. 2007; www.minitab.com).

Wolbachia Diagnosis and Strain Identification

DNA from individual rootworm adults was screened for infection using the 99 F and 994R 16S rRNA primers to amplify an \approx 800-bp band specific to *Wolbachia* (O'Neill et al. 1992, Giordano et al. 1997, Clark et al. 2001b). All diagnostic reactions had both a positive control using DNA from beetles of a *Wolbachia*-infected nondiapause laboratory colony (Giordano et al. 1997) and a negative control with molecular-grade water instead of DNA. Because the 99 F/994R primers tended to give a high rate of false negatives in PCR reactions for the DNA samples from Nebraska, especially those at concentrations lower than 100 ng/ μ l, two additional sets of *Wolbachia*-specific primers, *ftsZf1/ftsZr1* (\approx 1,050 bp; Werren 1997) and *wsp81F/wsp691R* (\approx 600 bp; Jeyaprakash and Hoy 2000), were used, respectively, to verify the negatives diagnosed by primers 99 F/994R.

To identify the *Wolbachia* strain infecting beetle populations from Nebraska, the PCR amplicons (*ftsZf1/ftsZr1*, \approx 960 bp and *wsp81 F/wsp691R*, \approx 600 bp) from single-beetle DNA samples were sequenced in both directions by using a GenomeLab DTCS kit and a CEQ 8000 DNA Analyzer (Beckman Coulter). The *ftsZ* and *wsp* PCR products were purified from 0.5% agarose gel with an Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Little Chalfont, United Kingdom). For each population from Nebraska, two to three individual DNA samples were randomly selected and sequenced. Sequences obtained in this study and previous publications (Giordano et al. 1997, Clark et al. 2001b, Segura-Leon 2004, Roehrdanz and Levine 2007) were aligned and compared using the BioEdit 7 software (Hall 1999).

Results***Genetic Diversity, HWE, and Linkage Disequilibrium***

Population genetic diversity, as measured by the number of alleles and expected heterozygosity, indicates that the DVV-D8 locus was the most polymorphic marker, whereas loci DVV-D9 and DVV-T3 were the least polymorphic (Table 1). The average number of alleles over the 11 markers ranged from 4.7 to 6.7 per locus and did not vary significantly among the populations ($F = 1.02$, $df = 4$, $P > 0.05$). Among the five populations, the expected heterozygosity (H_e) over the 11 loci ranged from 0.590 to 0.685 per locus. The observed heterozygosity (H_o) varied significantly among the populations ($0.501 \leq H_o \leq 0.736$, $F = 4.09$, $df = 4$, $P < 0.01$; Tukey's pairwise comparison, Safford, Clay Center, Mead > Polk and Stromsburg. The overall analysis, including the 11 loci from five populations, indicated that the genotypic frequencies did not conform to HWE ($P < 0.0001$) due to heterozygote deficits at

Table 1. Number of alleles (n), observed and expected heterozygosities (H_o and H_e), and inbreeding coefficients (F_{is}) of 11 microsatellite markers in five populations of western corn rootworm from Nebraska and Arizona

Lo-cus	Polk, NE				Stromsburg, NE				Mead, NE				Clay Center, NE				Stafford, AZ			
	n	H_o	H_e	F_{is}	n	H_o	H_e	F_{is}	n	H_o	H_e	F_{is}	n	H_o	H_e	F_{is}	n	H_o	H_e	F_{is}
DVV-D2	6	0.778	0.732	-0.051	6	0.767	0.716	-0.061	8	0.826	0.775	-0.056	7	0.800	0.756	-0.047	10	0.853	0.716	-0.177
DVV-D4	6	0.477	0.728	0.354 [†]	6	0.512	0.715	0.295 [†]	7	0.837	0.763	-0.086	7	0.781	0.742	-0.039	9	0.900	0.822	-0.078*
DVV-D5	3	0.362	0.371	0.035*	3	0.208	0.281	0.269	3	0.286	0.317	0.108 [†]	3	0.340	0.340	0.010	3	0.477	0.549	0.141 [†]
DVV-D8	10	0.643	0.804	0.212**	13	0.632	0.801	0.224**	14	0.800	0.888	0.110	14	0.933	0.877	-0.053 [†]	14	0.913	0.875	-0.033
DVV-D9	2	0.417	0.486	0.153	2	0.271	0.291	0.081	7	0.531	0.483	-0.088	3	0.600	0.494	-0.205	3	0.351	0.374	0.074
DVV-D10	4	0.551	0.627	0.132	4	0.469	0.586	0.209	7	0.780	0.790	0.001**	7	0.763	0.771	0.023	9	0.788	0.871	0.110**
DVV-D11	6	0.638	0.700	0.098	6	0.708	0.737	0.050	7	0.735	0.744	0.022	7	0.740	0.745	0.016	7	0.769	0.613	-0.243
DVV-T2	3	0.422	0.603	0.310**	3	0.313	0.547	0.437 [†]	4	0.659	0.646	-0.008*	4	0.707	0.563	-0.245	5	0.829	0.760	-0.075
DVV-T3	2	0.468	0.449	-0.032	2	0.408	0.425	0.050	3	0.612	0.507	-0.199	2	0.560	0.471	-0.179	4	0.700	0.584	-0.187
Dbal05	4	0.513	0.721	0.301**	4	0.500	0.735	0.331**	4	0.585	0.727	0.207	4	0.675	0.739	0.099	5	0.714	0.658	-0.071
Dbal07	3	0.750	0.662	-0.120	3	0.725	0.662	-0.082	4	0.773	0.692	-0.105	3	0.744	0.663	-0.111	5	0.800	0.718	-0.099
Avg.	4.5	0.547	0.626	0.137 [†]	4.7	0.501	0.590	0.162 [†]	6.2	0.675	0.666	-0.004	5.5	0.695	0.652	-0.056*	6.7	0.736	0.685	-0.059*

* $P < 0.05$, ** $P < 0.01$, † $P < 0.001$

six loci (DVV-D4, DVV-D5, DVV-D8, DVV-D10, DVV-T2, and DbA05; $P < 0.05$) and in four populations (Polk, Stromsburg, Clay Center, and Safford; $P < 0.05$). The likelihood and probability tests revealed that 12 and 16 of 275 (five populations \times 55 pairs among the 11 loci) possible pairs (4.4–5.8%) exhibited a low level of linkage disequilibrium among the 11 loci that were scored.

Population Genetic Structure and Isolation by Distance

Low, but significant, genetic differentiation was detected between Nebraska and Arizona populations ($F_{ST} = 0.059$, $P < 0.01$) and between the resistant (Polk and Stromsburg) versus susceptible (Mead and Clay Center) populations within Nebraska ($F_{ST} = 0.016$, $P < 0.01$) (Tables 2 and 3). No significant genetic differentiation was detected between populations within the resistant and susceptible groupings (Polk versus Stromsburg, $F_{ST} = -0.0055$, $P > 0.05$; Mead versus Clay Center, $F_{ST} = 0.002$, $P > 0.05$). Although isolation by distance was significant among all five populations ($R^2 = 71.2\%$, $P < 0.001$), the model did not hold for the four Nebraska populations ($R^2 = 0.0\%$, $P > 0.05$) (Fig. 2), indicating little distance effect on their genetic structure within the state. With high bootstrap values ($>50\%$), Polk and Stromsburg, Mead, and Clay Center were grouped, respectively, among the four Nebraska populations, whereas the population from Safford, Arizona, was clearly more distantly related to the Nebraska populations than the latter were to each other. (Fig. 3).

Table 2. F_{ST} estimates of western corn rootworm populations from Nebraska and Arizona

Locus	Among all 5 pop	Among 4 Nebraska pop	Between Nebraska and Arizona	Between Polk- Stromsburg and Mead–Clay Center
DVV-D2	–0.002*	–0.005	0.006**	0.000
DVV-D4	0.027***	–0.005	0.090***	0.000
DVV-D5	0.026***	0.003*	0.063**	0.003
DVV-D8	0.019***	0.022***	0.007	0.039***
DVV-D9	0.036*	0.038***	0.015	0.003***
DVV-D10	0.068***	0.041***	0.106***	0.064***
DVV-D11	0.019***	0.012***	0.027***	0.024***
DVV-T2	0.043***	0.016**	0.087***	0.026***
DVV-T3	0.047***	0.001	0.121***	0.008
DbA05	0.040***	–0.012	0.120***	–0.005
DbA07	0.001**	–0.006	0.017***	–0.003
Overall	0.029**	0.010**	0.059**	0.016**

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3. Pairwise F_{ST} estimates between five western corn rootworm populations

	Polk	Stromsburg	Clay Center	Mead
Stromsburg	0.0020			
Clay Center	0.0106**	0.0140**		
Mead	0.0139**	0.0216**	0.0055	
Safford	0.0721**	0.0820**	0.0522*	0.0440*

* $P < 0.05$, ** $P < 0.01$

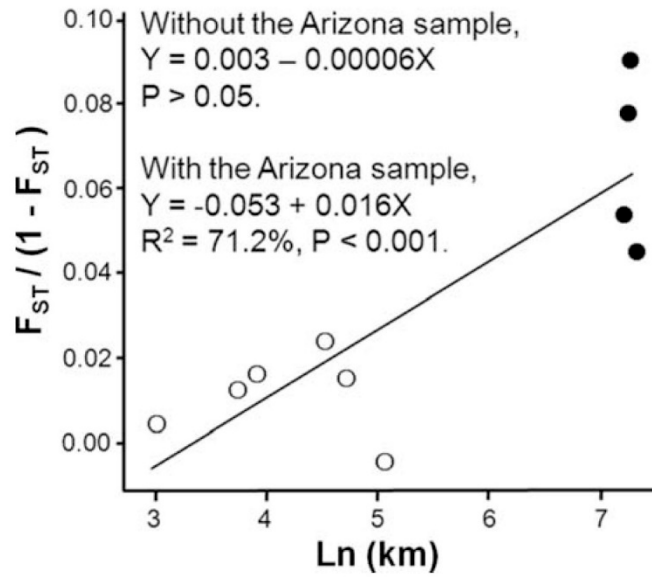


Figure 2. Linear regression of $F_{ST}/(1 - F_{ST})$ on distance (Ln km). The circles refer to coordinates within Nebraska, and the black dots represent comparisons between Nebraska and Arizona.

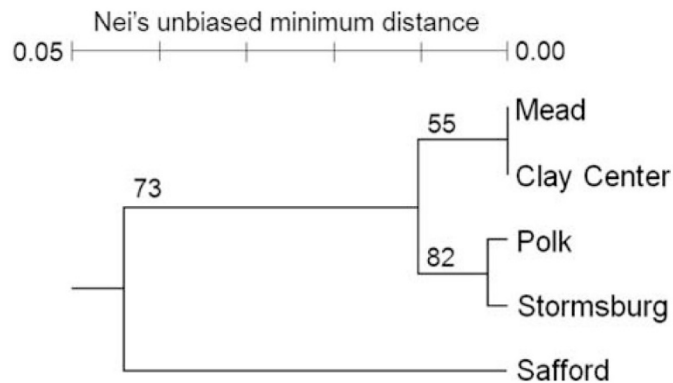


Figure 3. A dendrogram by the unweighted pair-group method with arithmetic average, showing genetic divergence among the populations. The numbers at the nodes indicate the bootstrap percentages >50%.

Correlations between Observed Heterozygosities and Mortalities by Insecticides

For both aldrin and methyl-parathion, mortality determined by diagnostic bioassays (Table 4) was significantly and positively correlated with the average observed heterozygosities (Table 1) in the populations (for aldrin, $r = 0.863$, $P < 0.001$; for methyl-parathion, $r = 0.840$, $P < 0.001$), respectively. Such a significant correlation between observed heterozygosity of a single locus and mortality also existed at DVV-D4, DVV-D8, DVV-D10, and DVV-T2 for aldrin and at DVV-D2, DVV-T2, DVV-T3, DbA05, and DbA07 for methyl-parathion across the populations ($r = 0.887$ – 0.966 , $P < 0.05$).

Table 4. Mortalities (%) of western corn rootworm adults to diagnostic concentrations aldrin and methyl parathion in the western corn rootworm populations (mean \pm SE; $n = 10$)

Pop	Aldrin	Methyl-parathion
Polk	10.9 \pm 4.2	36.0 \pm 6.8
Stromsburg	8.2 \pm 4.0	20.0 \pm 6.2
Clay Center	72.2 \pm 5.3	57.1 \pm 4.0
Mead	81.1 \pm 2.3	60.0 \pm 5.7
Safford	62.5 \pm 5.3	100.0 \pm 0.0

Wolbachia Infection

Wolbachia was not detected in the population from Safford, Arizona, but it was present in all individuals of the four Nebraska populations. Identical *FtsZ* and *wsp* sequences (Table 5) were found in all sequenced individual beetles, indicating a single strain of *Wolbachia* infecting the Nebraska populations and a lack of cytoplasmic incompatibility within and between Nebraska populations. Compared with *Wolbachia* sequences previously identified from *D. v. virgifera* adults and deposited in GenBank (Table 5), only 3-bp differences for *FtsZ* (\approx 960 bp) and only 1-bp difference in the (\approx 600 bp) *wsp* fragments were observed.

Table 5. Polymorphic sites in *ftsZ* and *wsp* sequences of *Wolbachia* in western corn rootworms

GenBank ID	Collection site	<i>ftsZ</i> sequence position ^a			<i>wsp</i> sequence position ^a	Reference
		544	545	865	347	
AF011270	Moriarty, NM; Blacksburg VA; Urbana, IL	T	G	G		Giordano et al. (1997)
AF011271						
U83108						
AY007552	Not specified	G	A	G		Clark et al. (2001)
AY136551	Gresham, NE	G	A	A		Roehrdanz and Levine (2007)
NA	Stromsburg, NE	T	G	G		Segura-Leon (2004)
NA	Polk, Stromsburg, Mead, Clay Center, NE	G	A	G		Sequenced in this study
AY138260	Brookings, SD				G	Roehrdanz and Levine (2007)
AY138261	Gresham, NE				A	Roehrdanz and Levine (2007)
DQ091306 to DQ091309	Urbana, IL; Clovis, NM; Freeville, NY; Texas Co., OK				G	NA ^b
NA	Polk, Stromsburg, Mead, Clay Center, NE				G	Sequenced in this study

a. Referenced to the positions in AY136551 of *ftsZ* and in the *wsp* sequenced in this study.

b. NA, not available.

Discussion

Analysis of microsatellite markers from western corn rootworms revealed significant genetic differentiation between populations with high levels of resistance (Polk and Stromsburg) relative to susceptible populations (Saunders and Clay), with a moderate loss of genetic diversity in the more resistant populations (15–32% lower H_o in Polk and Stromsburg; Table 1). A significant correlation between the observed heterozygosities and insecticide susceptibility, based on diagnostic bioassays, suggests that selection for insecticide resistance has shaped the genetic structure of western corn rootworm populations in Nebraska. However, it was not possible to determine the relative influence of organophosphate versus cyclodiene resistance because the Polk and Stromberg populations were highly resistant to both insecticide classes. The reduction of genetic diversity in the more resistant populations seemed to be genome-wide, but with various intensities at different loci. The lowest and highest losses of genetic diversity were 14.2% at DVV-D4 and DbA-07 and 42.9% at DVV-D10, respectively, as measured by the average number of alleles; and 2.8% at DbA-07 and 46.2% at DVV-T2 as revealed by the observed heterozygosities.

The positive correlations between observed heterozygosity and insecticide susceptibility may be attributable to a bottleneck experienced during selection for resistance during the time when the insecticides were driving local populations to low numbers. Such a bottleneck would affect genetic diversity across neutral loci, resulting in the observed patterns of correlation. The estimated gene flow between the York County populations (Polk or Stromsburg) and the two more susceptible populations (Clay Center or Mead) ranged between 1.5 and 2.0 individual per generation, limited enough to prevent restoration of diversity in York County after cessation of selection from cyclodiene insecticides or perhaps more likely, the later aerial applications of organophosphates that maintained low populations and therefore, genetic bottlenecks. An alternative explanation for the positive correlations between observed heterozygosities and insecticide susceptibility is a hitchhiking effect associated with the insecticide selections whereby microsatellite allele frequencies change due to selection operating upon linked genes that confer insecticide resistance. However, given that the pattern was observed across several loci, such an explanation seems unlikely.

Overall, the 11 microsatellite loci in the resistant populations lost 15–32% genetic diversity compared with the susceptible populations in Nebraska. A reexamination of recent studies of *D. v. virgifera* population genetics that used microsatellite markers (Kim and Sappington 2005a, Miller et al. 2006, Ciosi et al. 2008b) indicates that genetic diversity is also reduced in populations from the Corn Belt and Europe relative to populations derived from the southwestern United States and Mexico. The latter may be more representative of the purported area of origin (Melhus et al. 1954, Krysan and Smith 1987). Given that there is no evidence of a genetic bottleneck in North American populations, as inferred from rDNA internal transcribed spacer 1, mitochondrial DNA, and microsatellite markers (Szalanski et al. 1999, Kim and Sappington 2005b), several factors may contribute to reduced genetic diversity, including insecticide selection as revealed by the current study.

Although resistance to cyclodiene insecticides in western corn rootworm has persisted among populations throughout the Corn Belt (Metcalf 1986), considerable variation in resistance levels among field populations has been observed (Parimi et al. 2003; B.D.S., unpublished data). Moreover, cyclodiene resistance may be declining in frequency, intensity, or both in certain areas. From 1952 to 1961, aldrin at the rate of 2.24 kg (AI)/ha (equivalent to 0.5 ppm in top soil of 16.7 cm in depth per application) was recommended and used as a soil insecticide to control rootworms in Nebraska (Ball 1983). Considerable residues of aldrin and its epoxide, dieldrin, were consistently detected in Nebraska soil for several years after soil treatments were discontinued. However, the half-life of <2.5 yr for aldrin and \approx 5 yr for dieldrin in temperate soil (Edwards 1966, IPCS 1989) suggests a significant loss of residues over time, resulting in reduced selective pressures. Ball (1983) reported that the maximum residues of aldrin and dieldrin had decreased to 0.02 and 0.31 ppm, respectively, in Nebraska soil samples by 1973. More recent analyses from Nebraska soils in 2008 and 2009 suggest soil residues are <0.002 ppm for aldrin and 0.006 ppm for dieldrin (B.D.S., unpublished data). Although cyclodiene soil residues may have caused selection even after their use was discontinued, it seems unlikely that the high-level resistance observed among rootworms can be explained solely on the basis of exposure to soil residues. Relative to the concentrations (>0.5 ppm) that initially selected for resistance in rootworms

(Ball 1983), it is likely that the selection posed by the current soil residues should have been much reduced.

For methyl-parathion resistance, the distribution of resistant populations is generally correlated with areas where aerial application to suppress adult populations had been intensively practiced for >10 yr (Zhou et al. 2002). Although both the intensity and geographic range of resistance in rootworms has changed over time, probably due to movement of resistant beetles into areas where adult management had not been practiced (Parimi et al. 2003), populations susceptible to methyl-parathion still exist in proximity to resistant populations (Meinke et al. 1998, Miller et al. 2009).

Although the relative roles of initial selection for resistance and the subsequent changes in selection associated with alternative management approaches, our results support the general conclusion that insecticide selection has the potential to shape the genetic structure of rootworm populations. Moreover, these results suggest strongly that such fitness disadvantages associated with resistance to cyclodiene and methyl-parathion are relatively minor. Evidence for selection against resistant phenotypes is inferred from changes in the frequency of resistance in natural populations after changing patterns of insecticide use (Metcalf 1990). For both cyclodiene and methyl-parathion resistance, such changes seem to be exceedingly slow but may be contributing to the genetic structure observed in this study. Alternatively, the genetic structuring observed in Nebraska may be a remnant of genetic bottlenecks experienced during selection for resistance and a low rate of immigration from susceptible populations.

Cytoplasmic incompatibility caused by *Wolbachia* infection has been suggested to be primary factors both in speciation between the infected western corn rootworm and uninfected Mexican corn rootworms (Szalanski et al. 1999) and in separation of northern corn rootworm populations infested by two different strains (Roehrdanz and Levine 2007). The absence of *Wolbachia* infection in the Arizona population is consistent with results from a previous study (Giordano et al. 1997). The identical sequences of fragments of *ftsZ* and *wsp* genes suggest that the same strain of *Wolbachia* infects all the populations from Nebraska sampled in this study. Given the infection of all beetles sampled, the reproductive incompatibility caused by *Wolbachia* (Giordano et al. 1997) should neither disrupt random mating within the populations nor be a breeding barrier among the Nebraskan populations. Therefore, *Wolbachia* infection can be ruled out as a factor influencing the levels of insecticide resistance between beetle populations and causing the genetic differentiation between the resistant and susceptible populations of western corn rootworm. These results also support the conclusions of Ciosi et al. (2008) who reported that Arizona rootworm populations are genetically distinct from other Corn Belt populations. The absence of *Wolbachia* infection from the Arizona population and complete infection of Nebraska populations may be just as important as the large geographic distance that separates them. Giordano et al. (1997) proposed that *Wolbachia* infection for *D. virgifera* spp. probably originated in northwest Mexico or in New Mexico or western Texas. It is unclear how the Arizona populations became free of *Wolbachia* infection and a more thorough examination of populations from the southwestern United States is needed to gain a better understanding of genetic structure and *Wolbachia* infection.

Our results support that genetic differentiation exists between western corn rootworm populations that are resistant and susceptible to both aldrin and methyl-parathion in Nebraska and between the rootworm populations from Nebraska and Arizona. Within Nebraska, such genetic differentiation between the resistant and susceptible populations may be the result of insecticide selection because other factors such as isolation by distance, geographic barriers to gene flow, and reproductive incompatibility due to *Wolbachia* infection do not exist for the studied populations. Between Nebraska and Arizona, isolation by distance seems to contribute the most to the genetic structuring of the beetle populations and may be enhanced by the *Wolbachia*-induced incompatibility. Such information may provide insight into the evolution of insecticide resistance in western corn rootworm.

Acknowledgments – R. Norton (University of Arizona) provided samples from Safford, Arizona. We thank D. Taylor and D. Brinkerhoff (USDA-ARS) for assistance in microsatellite genotyping and scoring. N. Miller (University of Nebraska–Lincoln) provided critical review of an earlier draft of the manuscript.

References

- Ball, H. J. 1983. Aldrin and dieldrin residues in Nebraska (USA) soils as related to decreasing LD50 values for the adult western corn rootworm (*Diabrotica virgifera*), 1962–1981. *J. Environ. Sci. Health B* 18: 735–744.
- Ball, H. J., and G. T. Weekman. 1962. Insecticide resistance in the adult western corn rootworm in Nebraska. *J. Econ. Entomol.* 55: 439–441.
- Ball, H. J., and G. T. Weekman. 1963. Differential resistance of corn rootworms to insecticides in Nebraska and adjoining states. *J. Econ. Entomol.* 56: 553–555.
- Chen, H., M. Rangasamy, S. Y. Tan, H. Wang, and B. D. Siegfried. 2010. Evaluation of five methods for total DNA extraction from western corn rootworm beetles. *PloS ONE* 5: e11963. (doi: 10.1371/journal.pone.0011963).
- Ciosi, M., S. Toepfer, H. Li, T. Haye, U. Kuhlmann, H. Wang, B. Siegfried, and T. Guillemaud. 2008a. European populations of *Diabrotica virgifera virgifera* are resistant to aldrin, but not to methyl-parathion. *J. Appl. Entomol.* 133: 307–314.
- Ciosi, M., N. J. Miller, K. S. Kim, R. Giordano, A. Estoup, and T. Guillemaud. 2008b. Invasion of Europe by the western corn rootworm, *Diabrotica virgifera virgifera*: multiple transatlantic introductions with various reductions of genetic diversity. *Mol. Ecol.* 17: 3614–3627.
- Clark, T. L., L. J. Meinke, and J. E. Foster. 2001a. Molecular phylogeny of *Diabrotica* beetles (Coleoptera: Chrysomelidae) inferred from analysis of combined mitochondrial and nuclear DNA sequences. *Insect Mol. Biol.* 10: 303–314.
- Clark, T. L., L. J. Meinke, S. R. Skoda, and J. E. Foster. 2001b. Occurrence of *Wolbachia* in selected diabroticite (Coleoptera: Chrysomelidae) beetles. *Ann. Entomol. Soc. Am.* 94: 877–885.
- Coates, B. S., N. J. Miller, D. V. Sumerford, T. W. Sappington, B. D. Siegfried, and L. C. Lewis. 2009. Comparative performance of single nucleotide polymorphism (SNP) and microsatellite markers for the detection of population differentiation in *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). *J. Hered.* 100: 556–564.

- Degrugillier, M. E., S. S. Degrugillier, and J. J. Jackson. 1991. Nonoccluded, cytoplasmic virus particles and Rickettsia-like organisms in the testes and spermathecae of *Diabrotica virgifera*. *J. Invertebr. Pathol.* 57: 50–58.
- Eben, A., and A. Espinosa de los Monteros. 2004. Ideas on the systematics of the genus *Diabrotica* Wilcox and other related leaf beetles, pp. 59–73. *In* P. Jolivet, J. A. Santiago-Blay, and M. Schmitt (eds.), *New developments in the biology of Chrysomelidae*. SPB Academic Publishing, The Hague, The Netherlands.
- Edwards, C. A. 1966. Insecticide residues in soils. *Res. Rev.* 13: 83–132.
- Gillespie, J. J., K. M. Kjer, E. G. Riley, and D. W. Tallamy. 2004. The evolution of cucurbitacin pharmacophagy in rootworms: insight from *Luperini paraphyly*, pp. 37–57. *In* P. Jolivet, J. A. Santiago-Blay, and M. Schmitt (eds.), *New developments in the biology of Chrysomelidae*. SPB Academic Publishing, The Hague, The Netherlands.
- Giordano, R., J. J. Jackson, and H. M. Robertson. 1997. The role of *Wolbachia* bacteria in reproductive incompatibilities and hybrid zones of *Diabrotica* beetles and *Gryllus* crickets. *Proc. Natl. Acad. Sci. U.S.A.* 94: 11439–11444.
- Goudet, J. 1995. FSTAT version 1.2: a computer program to calculate F-statistics. *J. Hered.* 86: 485.
- Gray, M. E., T. W. Sappington, N. J. Miller, J. Moeser, and M. O. Bohn. 2009. Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. *Annu. Rev. Entomol.* 54: 303–321.
- Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment [ed.], and analysis program for Windows 95/98/NT. *Nucleic Acids. Symp. Ser.* 41: 95–98.
- Hartl, D. L., and A. G. Clark. 2007. *Principles of population genetics*, 4th ed. Sinauer, Sunderland, MA.
- [IPCS] International Programme on Chemical Safety. 1989. Environmental Health Criteria 91. Aldrin and Dieldrin. International Programme on Chemical Safety. (<http://www.inchem.org/documents/ehc/ehc/ehc91.htm>).
- Jeyaprakash, A., and M. A. Hoy. 2000. Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.* 9: 393–405.
- Kim, K. S., and T. W. Sappington. 2005a. Genetic structuring of western corn rootworm (Coleoptera: Chrysomelidae) populations in the US based on microsatellite loci analysis. *Environ. Entomol.* 34: 494–503.
- Kim, K. S., and T. W. Sappington. 2005b. Polymorphic microsatellite loci from the western corn rootworm (Insecta: Coleoptera: Chrysomelidae) and cross-amplification with other *Diabrotica* spp. *Mol. Ecol. Notes* 5: 115–117.
- Kim, K. S., S. T. Ratcliffe, B. W. French, L. Liu, and T. W. Sappington. 2007. Utility of EST-Derived SSRs as population genetics markers in a beetle. *J. Hered.* 99: 112–124.
- Kim, K. S., B. W. French, D. V. Sumerford, and T. W. Sappington. 2008a. Genetic diversity in laboratory colonies of western corn rootworm (Coleoptera: Chrysomelidae), including a nondiapause colony. *Environ. Entomol.* 36: 637–645.
- Kim, K. S., U. Stolz, N. J. Miller, E. R. Waits, T. Guillemaud, D. V. Sumerford, and T. W. Sappington. 2008b. A core set of microsatellite markers for western corn rootworm (Coleoptera: Chrysomelidae) population genetics studies. *Environ. Entomol.* 37: 293–300.
- Krysan, J. L., and R. F. Smith. 1987. Systematics of the *virgifera* species group of *Diabrotica* (Coleoptera: Chrysomelidae: Galerucinae). *Entomography* 5: 375–484.

- Krysan, J. L., I. C. McDonald, and J. H. Tumlinson. 1989. Phenogram based on allozymes and its relationship to classical biosystematics and pheromone structure among eleven diabroticines (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 82: 574–581.
- McKenzie, J. A. 1996. Ecological and evolutionary aspects of insecticide resistance. R. G. Landes Academic, Austin, TX.
- Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1997. Western corn rootworm resistance to insecticides: current situation in Nebraska. *Proc. Ill. Agric. Pestic. Conf.* 88–92.
- Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. *J. Econ. Entomol.* 91: 594–600.
- Meinke, L. J., T. W. Sappington, D. W. Onstad, T. Guillemaud, N. J. Miller, J. Komaromi, N. Levay, L. Furlan, J. Kiss, and F. Toth. 2009. Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) population dynamics. *Agric. For. Entomol.* 11: 29–46.
- Melhus, I. E., R. H. Painter, and F. O. Smith. 1954. A search for resistance to the injury caused by species of *Diabrotica* in the corn of Guatemala. *Iowa State Coll. J. Sci.* 29: 75–94.
- Metcalf, R. L. 1986. Foreword, pp. vii–xv. *In* J. L. Krysan and T. A. Miller (eds.), *Methods for the study of pest Diabrotica*. Springer, New York.
- Metcalf, J. A. 1990. Ecological and evolutionary aspects of insecticide resistance. Academic, London, United Kingdom.
- Miller, M. P. 1997. Tools for population genetic analyses (TFPGA) 1.3: a Windows program for the analysis of allozyme and molecular population genetic data. (Computer software distributed by author.)
- Miller, N. J., A. Estoup, S. Toepfer, D. Bourguet, L. Lapchin, S. Derridj, K. S. Kim, P. Reynaud, L. Furlan, and T. Guillemaud. 2005. Multiple transatlantic introductions of the western corn rootworm. *Science* 310: 992.
- Miller, N. J., K. S. Kim, S. T. Ratcliffe, A. Estoup, D. Bourguet, and T. Guillemaud. 2006. Absence of genetic divergence between western corn rootworms (Coleoptera: Chrysomelidae) resistant and susceptible to control by crop rotation. *J. Econ. Entomol.* 99: 685–690.
- Miller, N. J., M. Ciosi, T. W. Sappington, S. T. Ratcliffe, J. I. Spence, and T. Guillemaud. 2007. Genome scan of *Diabrotica virgifera virgifera* for genetic variation associated with crop rotation tolerance. *J. Appl. Entomol.* 131: 378–385.
- Miller, N. J., T. Guillemaud, R. Giordano, B. D. Siegfried, M. E. Gray, L. J. Meinke, and T. W. Sappington. 2009. Genes, gene flow and adaptation of *Diabrotica virgifera virgifera*. *Agric. For. Entomol.* 11: 47–60.
- Minitab, Inc. 2007. MINITAB 15 Statistical Software. Minitab, Inc., State College, PA.
- O'Neill, S. L., R. Giordano, A.M.E. Colbert, T. L. Karr, and H. M. Robertson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. U.S.A.* 89: 2699–2702.
- Oosterhout, C. V., W. F. Hutchinson, D.P.M. Wills, and P. Shipley. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* 4: 535–538.
- Parimi, S., M. E. Scharf, L. J. Meinke, L. D. Chandler, and B. D. Siegfried. 2003. Inheritance of methylparathion Resistance in Nebraska western corn rootworm populations (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 96: 131–136.

- Parimi, S., L. J. Meinke, B. W. French, L., D. Chandler, and B. D. Siegfried. 2006. Stability and persistence of aldrin and methyl-parathion resistance in western corn rootworm populations (Coleoptera: Chrysomelidae). *Crop Prot.* 25: 269–274.
- Pruess, K. P., J. F. Witkowski, and E. S. Raun. 1974. Population suppression of western corn rootworm by adult control with ULV malathion. *J. Econ. Entomol.* 67: 541–545.
- Raymond, M., and F. Rousset. 1995. An exact test for population differentiation. *Evolution* 49: 1280–1283.
- Roehrdanz, R. L., and E. Levine. 2007. *Wolbachia* bacterial infections linked to mitochondrial DNA reproductive isolation among populations of northern corn rootworm (Coleoptera: Chrysomelidae). *Ann. Entomol. Soc. Am.* 100: 522–531.
- Roselle, R. E., L. W. Anderson, R. G. Simpson, and M. C. Webb. 1959. Annual Report for 1959, Cooperative Extension Work in Entomology, Lincoln, NE.
- Roselle, R. E., L. W. Anderson, R. C. Simpson, and P. W. Bergman. 1960. Annual Report for 1960, Cooperative Extension Work in Entomology, Lincoln, NE.
- Roselle, R. E., L. W. Anderson, and P. W. Bergman. 1961. Annual Report for 1961, Cooperative Extension Work in Entomology, Lincoln, NE.
- Rousset, F. 1997. Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* 145: 1219–1228.
- Siegfried, B. D., and C. A. Mullin. 1989. Influence of alternative host plant feeding on aldrin susceptibility and detoxification enzymes in western and northern corn rootworms. *Pestic. Biochem. Physiol.* 35: 155–164.
- Segura-Leon, O. L. 2004. Phylogeography of *Diabrotica virgifera* LeConte and *Diabrotica virgifera zea* Krysan and Smith (Coleoptera: Chrysomelidae). Ph.D. Dissertation, University of Nebraska–Lincoln.
- Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* 87: 651–701.
- Sinkins, S. P., and S. L. O’Neill. 2000. *Wolbachia* as a vehicle to modify insect populations, pp. 271–287. In A. M. Handler and A. A. James (eds.), *Insect transgenesis: methods and applications*. CRC, Boca Raton, FL.
- Szalanski, A. L., and T. O. Powers. 1996. Molecular diagnostics of three *Diabrotica* (Coleoptera: Chrysomelidae) pest species. *J. Kans. Entomol. Soc.* 69: 260–266.
- Szalanski, A. L., R. L. Roehrdanz, D. B. Taylor, and L. Chandler. 1999. Genetic variation in geographical populations of western and Mexican corn rootworm. *Insect Mol. Biol.* 8: 519–525.
- Szalanski, A. L., R. L. Roehrdanz, and D. B. Taylor. 2000. Genetic relationship among *Diabrotica* species (Coleoptera: Chrysomelidae) based on rDNA and mtDNA sequences. *Fla. Entomol.* 83: 262–267.
- Tate, H. D., and O. S. Bare. 1946. Corn rootworms. *Nebr. Agric. Exp. Stn. Bull.* 381: 1–12.
- Waits, E., and U. Stolz. 2008. Polymorphic microsatellite loci from northern and Mexican corn rootworms (Insecta: Coleoptera: Chrysomelidae) and cross-amplification with other *Diabrotica* spp. *Mol. Ecol. Res.* 8: 707–709.
- Weir, B. S. 1990. *Genetic data analysis: methods of discrete population genetic data*. Sinauer, Sunderland, MA.
- Werren, J. H. 1997. Biology of *Wolbachia*. *Annu. Rev. Entomol.* 42: 587–609.

Zhou, X., M. E. Scharf, S. Parimi, L. J. Meinke, R. J. Wright, L. D. Chandler, and B. D. Siegfried, B. D. 2002. Diagnostic assays based on esterase-mediated resistance mechanisms in western corn rootworms (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 95: 1261–1266.