Role of a gamma-aminobutryic acid (GABA) receptor mutation in the evolution and spread of Diabrotica virgifera virgifera resistance to cyclodiene insecticides

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Abstract

The western corn rootworm, *Diabrotica virgifera virgifera*, is a damaging pest of cultivated corn that was controlled by applications of cyclodiene insecticides from the late 1940s until resistance evolved ~10 years later. Range expansion from the western plains into eastern USA coincides with resistance development. An alanine to serine amino acid substitution within the *Rdl* subunit of the gamma-aminobutyric acid (GABA) receptor confers resistance to cyclodiene insecticides in many species. We found that the non-synonymous single nucleotide polymorphism (SNP) G/T at the GABA receptor cDNA position 838 (G/T^{838}) of *D. v. virgifera* resulted in the alanine to serine change, and the codominant SNP allele T^{838} was genetically linked to survival of beetles in aldrin bioassays. A phenotypic gradient of decreasing susceptibility from west to east was correlated with higher frequencies of the resistance-conferring T^{838} allele in the eastern-most populations. This pattern exists in opposition to perceived selective pressures since the more eastern and most resistant populations probably experienced reduced exposure. The reasons for the observed distribution are uncertain, but historical records of the range expansion combined with the distribution of susceptible and resistant phenotypes and genotypes provide an opportunity to better understand factors affecting the species' range expansion.

Keywords: population genetics, range expansion, single nucleotide polymorphism, western corn rootworm, insecticide resistance.

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Introduction

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is arguably the single most damaging pest of field corn, *Zea mays* L., throughout most of the US corn belt (Sappington *et al*., 2006; Gray *et al*., 2009). Managing crop yield losses attributable to corn rootworm feeding damage is extremely difficult for several reasons, including the capacity of populations to evolve resistance to chemical insecticides (Metcalf, 1986; Meinke *et al*., 1998; Siegfried *et al*., 2005; Parimi *et al*., 2006; Gray *et al*., 2009; Meinke *et al*., 2009), corn plants expressing *Bacillus thuringiensis* (Bt) toxins (Gassmann *et al*., 2011) and cultural control practices, such as crop rotation (Levine *et al*., 2002; Gray *et al*., 2009). There are numerous examples of *D. v. virgifera* adaption to uniform, large-scale pest management practices that rely on chemical application (Meinke *et al*., 1998; Scharf *et al*., 2001) and cultural control (O'Neal *et al*., 2002).

Perhaps the most dramatic example of *D. v. virgifera* adaptation in terms of the area impacted and intensity was the selection for resistance to the cyclodiene insecticides. This class of compounds, with active ingredients benzene hexachloride (Muma *et al*., 1949), aldrin, chlordane (Ball & Hill, 1953) and heptachlor (Ball & Roselle, 1954), was commonly used as soil treatments for the control of larval *D. v. virgifera* feeding on corn roots from the late 1940s to the early 1960s. By 1959, almost 1 million kg of aldrin was used as a soil insecticide in Nebraska (Ball, 1983). Control

failures with these compounds were first noted in Nebraska in 1959 (Roselle *et al*., 1959), and further evaluations in 1960 (Roselle *et al*., 1960) and 1961 (Roselle *et al*., 1961) revealed the magnitude and rapid development of the resistance. The susceptibility of Nebraska field populations to aldrin and heptachlor was reduced as much as 1000-fold (Ball & Weekman, 1962, 1963), and provided direct evidence for resistance evolution. Because of their persistence and widespread environmental contamination, the US Environmental Protection Agency banned the use of cyclodiene insecticides in 1972. In spite of the discontinued use of these compounds, resistance levels remained high among most field populations (Parimi *et al*., 2006) although reduced resistance among field populations in Nebraska has been suggested (Ball, 1983).

Corn-growing areas affected by *D. v. virgifera* were limited to those west of the Missouri River prior to 1960, but in the second half of the 20th century, *D. v. virgifera* underwent an eastward range expansion that pushed the species distribution across most of the US corn belt by 1980 (Metcalf, 1983, 1986; Gray *et al*., 2009). *D. v. virgifera* reached the East Coast in the early 1990s (Meinke *et al*., 2009), and the alleles for cyclodiene resistance were apparently carried eastward by migrants involved in this range expansion (Parimi *et al*., 2006). Increased invasiveness of the population after selection for cyclodiene resistance was suggested to have resulted from an overall increased fitness and behavioural changes associated with the trait (Metcalf, 1986); however, the rapid eastward expansion may also be related to the increased environmental carrying capacity resulting from the adoption of continuous corn production practices in the eastern corn belt after the second world war (Gray *et al*., 2009; Meinke *et al*., 2009).

Multiple introductions of *D. v. virgifera* into various parts of Europe led to the invasion of a significant portion of central and southeastern Europe, northern Italy and Switzerland, southern Germany and eastern France (Miller *et al*., 2005; Ciosi *et al*., 2008). The invasive populations collected from nine different European corn fields showed high levels of resistance to aldrin (Ciosi *et al*., 2009), and the nearly fixed frequency of resistance did not vary significantly in either of the two independent outbreaks in central and southeastern Europe and northern Italy. The resistance trait in Europe is likely to have been introduced by the founding individuals from North America and may make cyclodiene resistance a useful marker for identifying the potential source population(s).

As a receptor for the major inhibitory neurotransmitter in insects, the gamma-aminobutyric acid [(GABA) γ-aminobutyric acid] receptors are ligand-gated chloride channels that regulate chloride flux and induce inhibitory post-synaptic graded potentials. GABA receptors are a target for a number of insecticides, including the cyclodienes (ffrench-Constant *et al*., 2000; Raymond-Delpech *et al*., 2005). Since a GABA-receptor subunit encoding a resistance-associated mutation (*Rdl*) was first isolated from a dieldrin resistant strain of *Drosophila melanogaster* (ffrench-Constant *et al*., 1993), *Rdl*-like mutations have been found in several other insect orders (ffrench-Constant *et al*., 2000). Cyclodiene resistance remains a model for genetic selection and the evolution of target-site-mediated resistance in a number of pest insects (ffrench-Constant *et al*., 2000), and its presence in *D. v. virgifera* populations provides an opportunity to examine the evolution of target-site-mediated resistance and subsequent dispersion of causal genes within natural populations (ffrench-Constant *et al*., 1993). The use of cyclodiene resistance traits as a tool to examine population genetics of resistance could be facilitated by the development of molecular genetic markers that can distinguish among resistant and susceptible phenotypes.

In the present report, we describe the development and validation of a molecular genetic marker to detect the *Rdl* mutation in *D. v. virgifera* populations. We also document significant variation in susceptibility to aldrin among North American *D. v. virgifera* populations and show that this variation is linked to the frequency of the *Rdl* mutation in the GABA receptor gene. We also demonstrate the existence of a geographical west-east gradient in both aldrin susceptibility and frequency of the *Rdl* mutation which may suggest a role for this mutation in the invasive nature of this species.

Results

Bioassays of field collections

Results of diagnostic bioassays involving exposure to a concentration of the cyclodiene, aldrin, corresponding to the LC_{99} of the susceptible non-diapause strain indicate significant local and regional variation in susceptibilities (Figs 1 and 2). Bioassays of rootworm adults from 2006 to 2011 from multiple locations showed that the most susceptible population (Colorado) exhibited a mortality rate of >90% while the most resistant populations (Pennsylvania) exhibited a mortality rate of <10%. The lowest mortality rates were detected among populations in the eastern portion of the species' range (Pennsylvania), which is consistent with previous results from dose–response assays and LD_{50} calculations (Parimi *et al*., 2006).

The only susceptible population to be identified by diagnostic bioassays was the non-diapause laboratory strain established from field collections in 1968 (Branson, 1976), but that was from an area where resistance was reported at the time of collection (Metcalf, 1986). Continual laboratory rearing has reduced the genetic variation within the non-diapause strain (Kim *et al*., 2007), and genetic drift may have resulted in loss of resistance alleles.

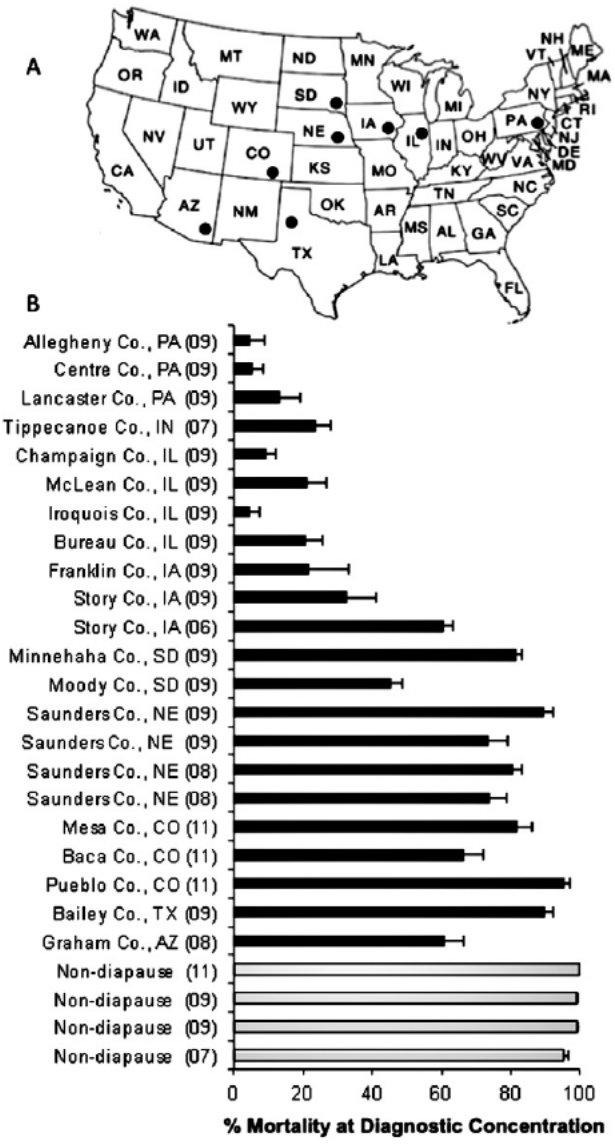

Figure 1. (A) Collection states and (B) mortality rates of adult *Diabrotica virgifera virgifera* to a diagnostic concentration of aldrin corresponding to the LC_{99} of the susceptible nondiapause strain (grey bars) from collection sites (counties and year of collection) across the USA. Each value represents the mean of at least five replications (10 beetles per replication) per population.

Variation in susceptibility was also observed among sites within close geographic proximity within the state of Nebraska (Fig. 2). Overall, beetles from collection sites in Nebraska exhibited greater susceptibility than beetles from the eastern USA; however, high levels of resistance were observed among three independent collections from Polk County, Nebraska (<10% mortality), which suggested that small-scale population structure may exist. These results confirm that cyclodiene resistance is still widely persistent in the field, although the resistance level and potentially the frequency of resistance-conferring alleles vary considerably.

Gamma-Aminobutyric Acid Receptor cDNA and Rdl *Identification*

Degenerate PCR primers were used to amplify a fragment of the GABA receptor gene from *D. v. virgifera*, and subsequent rapid amplification of cDNA ends (RACE) was used to obtain the complete cDNA sequence. The 1218-basepair (bp) cDNA sequence encodes a putative protein of 406 amino acids (GenBank accession KC288141). A BLASTX search of the National Center for Biotechnology Information (NCBI) nonredundant (nr) protein database, using the 1218-bp cDNA sequence as a query, indicated that the conceptual translation shares 91% identity with the GABA-gated chloride channel from *Oulema oryzae*, 89% identity with GABA receptor isoform B from *Tribolium-castaneum*, and ≥72% identity with GABA receptors from other insect species (Table S1 and Fig. S1). Protein structural predictions indicated three transmembrane domains (M1–M3), which are features common to ligand-gated ion channel proteins (Schofield *et al.*, 1987). Sequencing of GABA receptor cDNA from aldrin-resistant and -susceptible *D. v. virgifera* revealed a G to T point mutation at nucleotide position 838 in KC288141, which resulted in an alanine (A) to serine (S) amino acid substitution at position 280 in transmembrane region M2 (Fig. S1). This *D. v. virgifera* nonsynonymous mutation conforms to a conserved *Rdl* point mutation associated with cyclodiene resistance in other insect species (ffrench-Constant *et al.*, 2000; Buckingham *et al.*, 2005). A similar amino acid change confers phenotypic resistance by modifying cyclodiene binding to the GABA receptor *Rdl* subunit in a number of different insects (Zhang *et al.*, 1994), and suggests this mutation is the genetic basis for cyclodiene resistance in *D. v. virgifera*.

Bacterial artificial chromosome library screening and sequencing

To develop a genetic marker for PCR screening of *D. v. virgifera* for cyclodiene resistance using genomic DNA as template, bacterial artificial chromosome (BAC) clones encoding the gene were isolated. This was necessary because intron sequences in the *D. v. virgifera* genome are large (Coates *et al.*, 2012) and have been predicted to cause difficulty in the development of single nucleotide polymorphism (SNP) markers from expressed sequence tag (EST) sequences for this species (Coates *et al.*, 2009). PCR screening of DvvBAC1 with *D. v. virgifera* GABA receptor PCR primer pairs amplified the genomic sequence from positive control gDNA as well as nine library clones (listed in Fig. S2; remaining data not

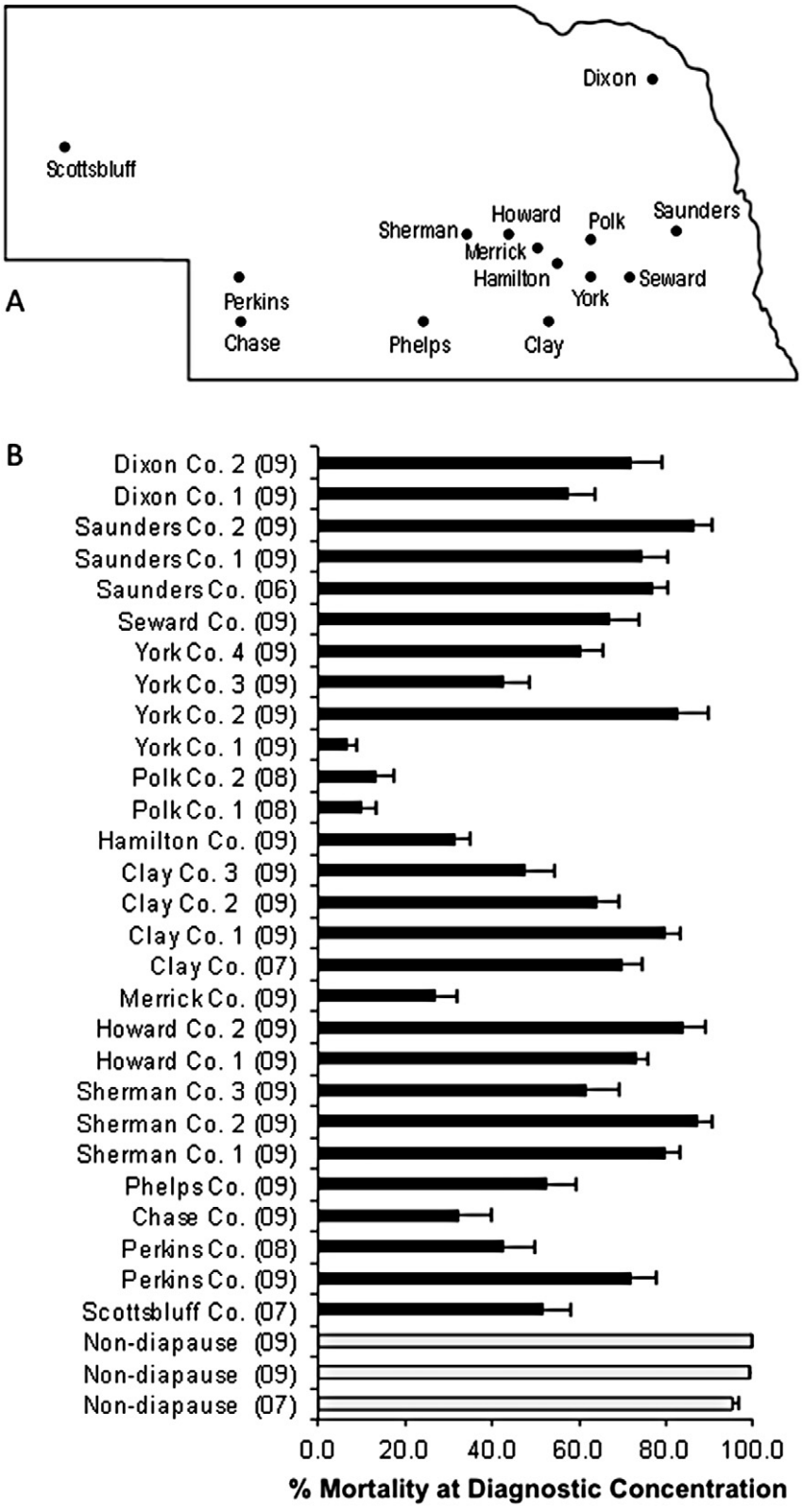

Figure 2. (A) Collection sites and (B) mortality rates of adult *Diabrotica virgifera virgifera* to a diagnostic concentration of aldrin corresponding to the LC_{99} of the susceptible nondiapause strain (grey bars) from collection sites (counties and year of collection) in Nebraska. Counties with more than one collection site in the same year represent different collection fields. Each value represents the mean of at least five replications (10 beetles per replication) per population.

Table 1. Summary of contigs per *Diabrotica virgifera virgifera* BAC containing a marker for GABA receptor assembled from Roche GS-FLX Titanium sequencing data

Mid-tag Library	BAC Clone	Marker	Raw data (reads/Mb)	Assembled data (reads/Mb)	GenBank accession	Mean (SD) Contig size (kb)
RL006	129I09	GABA	28 696/ 9.495	28 161/ 9.290	KC248068	21.8 (25.2)
RL007	222A09	GABA	20 807/ 7.035	20 416/ 6.885	KC248067	11.5 (5.5)
RL008	279 K02	GABA	24 326/ 4.264	24 307/ 3.404	KC248069	28.1 (28.5)
		Total	73 829/ 20.794	72 884/ 19.579		17.5 (17.6)

GABA, gamma-aminobutyric acid.

shown). Subsequent *Eco*RI and *Pst*I restriction fragment length polymorphism (RFLP) analysis of these nine DvvBAC1 clones indicated that the number of fragments ranged from 15 to 18 (mean [SD] 16.33 [1.22]), of which ≥78.9% were shared among clones. The BAC plasmid DNA from three clones, 129I06, 222A09 and 279 K02, was successfully used to generate mid-tag libraries RL06, RL07, and RL08, respectively. Next generation (Roch 454 GS FLX) sequencing generated 74 Mb of raw sequence data with mean (SD) read lengths of 281.3 (92.2) bp across libraries (reduced to 72.8 Mb after quality trimming with $q \geq 30.0$; Table 1). Assembly of read data resulted in contigs with a mean (SD; range) size of 17.5 (17.6; 2.3–72.9) kb. BLASTN searches identified positions 685 to 1243 of the GABA receptor cDNA sequence from KC288141 in all three clones (≥98% similarity; mean [SD] 139.3 [31.7] bp). Specifically, four cDNA regions corresponding to positions 685 to 822, 823 to 926, 927 to 1107, and 1108 to 1243, were flanked by the canonical AG[exon]GT splice sequence, encoded amino acid positions 185 to 370 (or 43.8% of the exon sequence; Fig. 3), and thus probably represent four exons of the GABA receptor. The locus for the *Rdl* mutation (SNP G/T^{838}) was identified within a 181-bp exon with 99% similarity to the same cDNA region, but was fixed for the G nucleotide among all three clones and was positioned 43 bp downstream of an intron/exon junction (Fig. 3). Detection of the cyclodiene susceptible allele within DvvBAC1 was expected, because the library was created from gDNA isolated from the susceptible non-diapause strain (Coates *et al*., 2012). Complete intron sequences were assembled only for a 5.0-kb intron between cDNA positions 926 and 927, whereas other genome regions between exons were not assembled intact. The intron regions that were assembled showed a maximum size of 48.8 kb (in contig 1 of DvvBAC1 clone 279 K02; Fig. 4), and were consistent with the large intron sizes observed among other DvvBAC1 clones (Coates *et al*., 2012) and other *D. v. virgifera* genes (Sayed *et al*., 2007).

Correlation of the T^{838} SNP with cyclodiene resistance

Using evidence from BAC sequence assemblies, a molecular assay was developed to genotype the G/T^{838} SNP locus. A single base extension assay (Black & Vontas, 2007) was successfully developed using the Beckman-Coulter GenomeLab SNP starter kit followed by separation on a CEQ 8000 sequencer (Beckman-Coulter, Brea, CA, USA). Among individuals from the nondiapause strain and field collections from Nebraska, Illinois, and Pennsylvania, the three genotypes, G/G^{838}, G/T^{838} and T/T^{838}, were present at cDNA position 838, and the T/T^{838} genotype was highly correlated with adult *D. v. virgifera* susceptibility based on survival in diagnostic bioassays (Fig. 5). Linear regression of T^{838} allele frequency was strongly and significantly correlated with mortality rate (percent T^{838} allele frequency = 90.37 – 0.944 x percent mortality, $P = 0.016$, $R^2 = 0.968$); thus, the results indicate increased resistance in field-collected rootworms is associated with increased frequencies of alleles with the T^{838} nucleotide (serine amino acid).

Association of the T^{838} allele with aldrin resistance is also indicated by paired bioassay and PCR-RFLP data. Of 71 adult *D. v. virgifera* from Ames, IA exposed to diagnostic concentrations of aldrin, 31 were unaffected, 26 dead, and 14 moribund. There was a strong and significant correlation between the unaffected phenotype and the presence of ≥1 T^{838} allele when compared with dead individuals ($r = 0.9077$; $N = 56$; $t = 16.042$; degrees of freedom = 55; $P = 2.5 \times 10^{-7}$). When PCR-RFLP genotypes from the moribund phenotype group were included, a significant correlation ($r = 0.601$) was observed between the number of T^{838} alleles and aldrin resistance ($N = 71$;

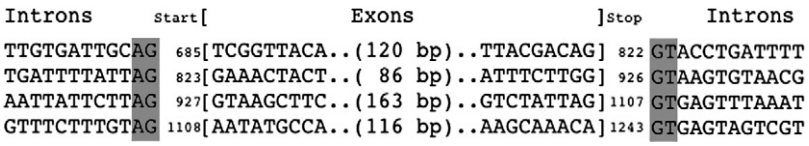

Figure 3. Annotation of gamma-aminobutyric acid (GABA) receptor intron splice junctions, where canonical AG and GT dinucleotides flank four exons within genomic regions assembled from 454 sequence data.

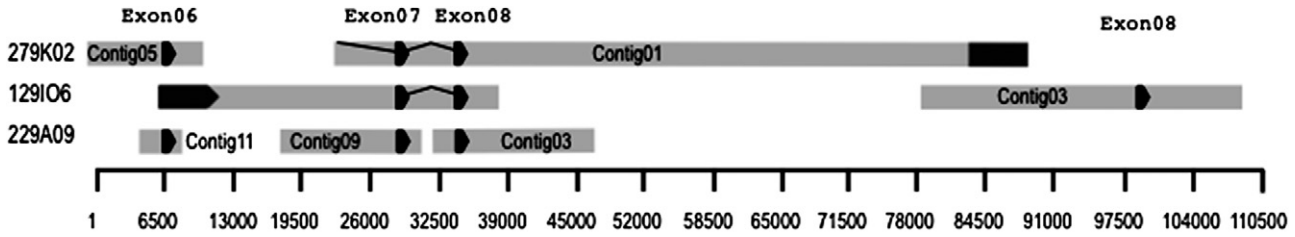

Figure 4. Consensus alignment of the *Diabrotica virgifera virgifera* gamma-aminobutyric acid (GABA) receptor gene sequence reconstructed from bacterial artificial chromosome clones that encode cDNA positions 685 to 1235. Arrows indicate direction of coding sequence in exons and vector sequence is indicated in dark grey. Physical distance indicated in base pairs along the bottom scale.

$t = 6.246$; degrees fo freedom = 69; $P < 0.001$). The role of the T^{838} SNP in conferring aldrin resistance was confirmed by the lack of the G/G^{838} genotype among beetles that survived exposure to the estimated LC_{99} concentration. Codominance of the T^{838} allele in conferring aldrin resistance is suggested by 13 of 44 resistant adults (29.5%) having a heterozygous G/T^{838} genotype (T^{838} allele frequency 0.852), while 16 of 27 (59.3%) dead beetles also were heterozygous.

Distribution of the T^{838} single nucleotide polymorphism in the North American population

Four of the six collection sites showed genetic variation for the *Rdl* mutation (G/T^{838} SNP locus; Kansas observed heterozygosity (H_O) = 0.375; Nebraska $H_O = 0.458$; Iowa $H_O = 0.583$; Ohio $H_O = 0.125$), whereas the New York and Delaware locations were fixed for the resistant T^{838} allele. There was no significant difference in expected heterozygosity (H_E) and H_O in any polymorphic population (All $P \geq 0.199$; data not shown). Pairwise F_{ST} estimates based on this locus showed significant levels of differentiation among 9 of the 15 comparisons using the Benjamini and Yekutieli (B-Y) adjusted threshold for multiple tests ($\alpha = 0.015$; Table 2). Analysis of molecular variance (AMOVA) that combined western populations (Kansas, Nebraska, Iowa) and eastern populations (Ohio, New York, Delaware) indicated that 35.5% of the variation was among groups and θ_{CT} (F_{ST}) = 0.355 ($P < 0.097 \div 0.009$).

Geographic distance and F_{ST} /$(1 - F_{ST})$ based upon genotypes at the G/T^{838} SNP locus were strongly and significantly correlated ($r^2 = 0.975$; Mantel test, $P = 0.002$; Fig. 6). This suggests that there may be west to east geographic cline of T^{838} allele frequency in the US *D. v. virgifera* population. These genotypic results agree with the phenotypic variation observed among sample location within the USA (Fig. 1). They further suggest that the SNP markers are suitable for associating genotype with phenotype.

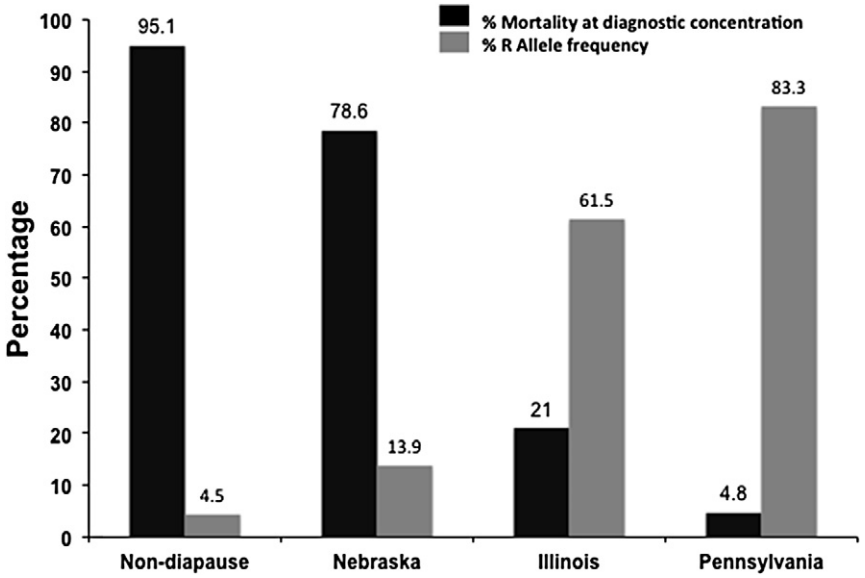

Figure 5. Correlation between resistance allele frequency in the *Diabrotica virgifera virgifera Rdl* gene, as determined by single base extension analysis, and susceptibility of adults from indicated populations based on % survival at a diagnostic aldrin concentration corresponding the LC_{99} of the susceptible nondiapause strain.

Table 2. Pairwise comparison of F_{ST} estimates between *Diabrotica virgifera virgifera* sample locations (below diagonal) and *P*-values of corresponding comparisons (above diagonal), based on the G/T^{838} SNP locus in the *Rdl* gene. Significant differences are those with *P*-values less than a Benjamini and Yekutieli adjusted $\alpha = 0.015$

	KS	NE	IA	OH	NY	DE
KS		0.991	<0.001	<0.001	<0.001	<0.001
NE	–0.021		<0.001	<0.001	<0.001	<0.001
IA	0.002	0.002	<0.001	<0.001	<0.001	<0.001
OH	0.301	0.301		0.225	0.225	0.198
NY	0.426	0.426	0.043			0.991
DE	0.726	0.426	0.043	0.000	0.000	<0.001

KS = Concordia, KS; NE = Mead, NE; IA = Ames, IA; OH = Bowersville, OH; NY = Cobleskill, NY; DE = New Deal, DE.

Discussion

The combined results of diagnostic bioassays and molecular analysis of cyclodiene resistance in *D. v. virgifera* confirm that increased resistance in field-collected rootworms is associated with increased frequencies of alleles with the T^{838} nucleotide (serine amino acid). Similar positive associations of the *Rdl* mutation with increased cyclodiene resistance have been observed among a variety of arthropod pests (reviewed by ffrench-Constant *et al.*, 2000; Bass *et al.*, 2004; Hansen *et al.*, 2005) and support the conclusion that cyclodiene resistance in rootworms is associated with increased frequencies of T^{838} alleles (serine amino acid). Results of one-to-one comparisons of genotype with resistance phenotype confirm survival by resistant homozygotes and some heterozygotes, and agree with our population-level results showing increased T^{838} allele frequencies in resistant field populations and that GABA receptor-based cyclodiene resistance is a codominant genetic trait (Georghiou, 1969; Beeman & Stuart, 1990).

We also identified considerable phenotypic and genotypic heterogeneity in resistance at both local and broader geographic scales, and a west-to-east cline in resistance frequency across the US corn belt states with higher resistance frequencies among eastern populations; however, the factors that contribute to shaping the genetic structure of these populations are uncertain. Such factors might include histories of selection pressure within the local region or genetic drift, although it appears unlikely that drift is an important factor given an absence of differentiation among geographically distinct populations (Miller *et al.*, 2009). If drift were contributing to the observed pattern of resistance, similar variation should be evident from population genetics analyses; however, a number of studies using neutral microsatellite (Kim & Sappington, 2005a, 2005b) and SNP markers (Coates *et al.*, 2009) suggest a general lack of differentiation across the North American distribution. This lack of differentiation may be related to a stratified dispersal (Hengeveld, 1989) that might have occurred during range expansion involving the coalescence of small colonizing populations established ahead of the advancing invasion front. This pattern of movement has been suggested to effectively negate genetic founder effects, which is again consistent with previous population genetics analyses showing a lack of differentiation among North American populations (Kim & Sappington, 2005a, 2005b; Coates *et al.*, 2009).

It is difficult to estimate the role of selection intensities, especially during the period when cyclodiene insecticides were routinely used for rootworm management in areas representing the front edge of the advancing invasion. Clearly, the local variation observed in Nebraska may be related to local differences in selection pressure (Chen *et al.*, 2012) that persisted in spite of the absence of insecticide use after 1972; however, the pattern of generally higher levels of resistance among eastern populations would appear to be in opposition to selection intensity, because *D. v. virgifera* moved into corn-growing regions of Indiana and eastward after ~1972 (Gray *et al.*, 2009) and subsequent to the U.S. Environmental Protection Agency ban on the use of cyclodiene insecticides; therefore, the highest frequencies of resistance were detected at the leading edge of the invasive front where selection intensity was likely to be reduced relative to the area of origin.

A number of studies have suggested that low-frequency alleles can sometimes 'surf' on the wave of a population

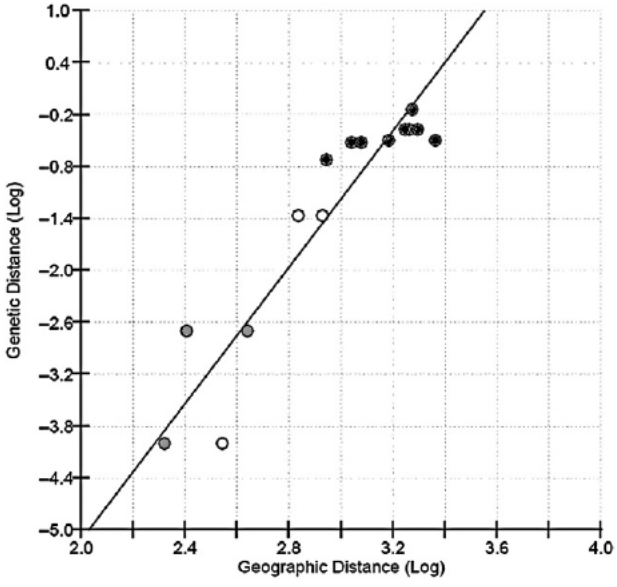

Figure 6. Regression of Log_{10} genetic distance (F_{ST} /(1 – F_{ST}), based on the G/T^{838} SNP locus in the *Rdl* gene) on Log_{10} geographic distance among *Diabrotica virgifera virgifera* populations. Correlations and probabilities were estimated from a Mantel test with 10000 bootstrap replications. Comparisons among Midwestern locations (Concordia, KS, Mead, NE, and Ames, IA) (◍), among eastern locations (Bowersville, OH, Cobleskill, NY and New Deal, DE (○), and between Midwestern and Eastern locations (●).

range expansion, such that the genetic make-up of the expanding population can rapidly change over time and space, leading to potentially large allele frequency differences (Edmonds *et al*., 2004; Klopfstein *et al*., 2005; Excoffier & Ray, 2008; Lehe *et al*., 2012). This 'surfing' hypothesis may be a contributing factor to the higher frequencies of the T^{838} allele and resistance that appear to be observed on the wave of the rootworm invasion front. A potentially simpler explanation is that the resistance allele had become fixed in the founding population and that the subsequent eastward spread was initiated by resistant homozygotes. However, estimates of resistance intensity along the invasion front (Hamilton, 1965) in Iowa and South Dakota indicate that a mixture of resistant and susceptible genotypes represented the eastward expansion, and the degree of resistance generally decreased towards the eastern limits of detection. As a consequence, the potential for resistance alleles to 'surf' along the wave of the expanding population resulting in large frequency increases may have contributed the near fixation of resistance in eastern populations.

Another contributory factor may be the potential for immigration from more western areas where selection intensity was likely reduced and where resistance never became fixed. Although potential fitness costs associated with cyclodiene resistance in western corn rootworm appear to be slight, based on the very slow decline in resistance frequency, the movement of susceptible alleles combined with a slight fitness deficit may be contributing to the slow but significant decline in resistance frequency among the most western population. This conclusion is generally supported by the higher susceptibility noted among collections obtained from Colorado which were generally the most susceptible and from the most western areas where field collections were obtained. Estimates of the fitness costs associated with cyclodiene resistance in other insects have varied. McKenzie (1996) reported relatively strong fitness costs in the sheep blowfly *Lucilia cuprina*, resulting in sharp declines in the frequency of resistance phenotype subsequent to the removal of dieldrin for blowfly control (Hughes and McKenzie, 1987); however, in the case of *D. melanogaster*, no fitness costs were apparent in population cage experiments with different strains at fixed temperature (Aronstein *et al*., 1995; ffrench-Constant *et al*., 2000). Although no direct measurements of fitness are available for cyclodiene-resistant western corn rootworms, the persistence of resistance among field populations would argue that such costs are likely to be slight but may play a role in the pattern of susceptibility noted among field populations.

Although the reasons for the observed distribution in resistant frequencies are uncertain, historical records of the *D. v. virgifera* range expansion, together with the distribution of cyclodiene insecticide susceptible phenotypes and genotypes in the USA, provide an opportunity to better understand factors affecting the species' range expansion. Moreover, the identification of variation in both resistance phenotype and genotype may provide an opportunity to better identify the source of invasive populations in Europe and better protect other corn-growing regions of the world at risk of invasion from this important pest species.

Experimental procedures

Field collection and bioassays

D. v. virgifera adults were collected either from corn plants or from weeds adjacent to corn fields from June to September in 2006–2011, in geographically distant sites in Nebraska, Illinois, Iowa, Indiana, Pennsylvania, Colorado, Arizona and Texas (Fig. 1; ≥60 unsexed adults per collection site). Adults from a nondiapause laboratory strain of *D. v. virgifera* were previously shown to be aldrin-susceptible (Parimi *et al*., 2006), and were obtained from the US Department of Agriculture (USDA) Agricultural Research Service (ARS), North Central Agricultural Research Laboratory, Brookings, SD for use as a susceptible control. The Nebraska samples were subjected to aldrin bioassays the same day as collected, whereas populations from other states were provided by cooperators on an artificial diet (Jackson, 1985) or a diet of fresh corn ears and shipped to the University of Nebraska-Lincoln by overnight delivery. All adults were maintained on fresh corn ears at ambient temperature (20–24 °C) for 24 h before aldrin bioassays.

Susceptibility of field populations was determined using diagnostic aldrin bioassays, which were based on the LC_{99} calculated from the susceptible non-diapause laboratory *D. v. virgifera* strain (Ciosi *et al*., 2009; Chen *et al*., 2012). In brief, 0.5 ml of reagent grade >99.5% pure acetone containing 16.5 μg aldrin (technical grade 98% AI, Sigma-Aldrich, St. Louis, MO, USA) was added to a 20-ml glass scintillation vial. Vials were placed on a commercial hot dog roller within a fume hood, and rolled for 18 min to evaporate the solvent and evenly coat the inner surface of the vial. Ten unsexed adults were bioassayed per vial for each population and replicated ≥5 times for each population, and one control vial (acetone only) was used per population. Vials were maintained at 22–24 °C and adult mortality (defined as a lack of coordinated movement; Parimi *et al*., 2006) was evaluated after a 24-h exposure.

cDNA preparation and sequencing

Total RNA was extracted from pools of adult *D. v. virgifera*, previously shown to be resistant based on diagnostic aldrin bioassays (Mead, Saunders County, NE, USA and an Indiana field population), and from the susceptible nondiapausing strain using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA extracts using the Omniscript RT Kit (50) (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. The degenerate PCR primer pair DF2 (5′-GAA GYT TCG GYT AYA CVA TGC-3′) and DR2 (5′-ACC ATA SAC GAA RCA DGT BCC CAG ATA-3′) was designed using Primer3 (Rozen & Skaletsky, 2000) to amplify an internal gene fragment based on predicted annealing to conserved motifs within insect GABA receptors from *T. castaneum* (GenBank accession S78567.1), *Apis mellifera* (GenBank accession AF094822.1), *Plutella*

xylostella (GenBank accession AY847747.1) and *D. melanogaster* (GenBank accession U02042.1). PCR was performed in a 10-μl volume containing 100 ng of cDNA, 1.5 units of *Taq* polymerase, 0.25 μl dNTP (10 mM), and 1 μl of the 5′ and 3′ primers (each at 10 μM) in 1X *Taq* polymerase buffer (50 mM KCl/10 mM Tris HCl, pH 8.3/1.5 mM $MgCl_2$, 0.01% gelatin) (Sigma-Aldrich). The DNA was amplified by an initial denaturation at 94 °C for 3 min, followed by 38 cycles of annealing at 59 °C for 45 s, extension at 72 °C for 1 min, and denaturation at 94 °C for 45 s, and finally by an extension at 72 °C for 10 min. PCR products were purified using Qiaquick PCR Spin Columns (Qiagen) and sequenced at University of Nebraska-Lincoln, Genomics Core Research Facility or the Genomics and Sequencing Laboratory, Research Instrumentation Facility, Auburn University. Sequence similarity to the GABA receptor was verified by a BLASTX search of the NCBI nr protein database.

Full-length cDNA was isolated by RACE, performed with the SMART RACE cDNA amplification kit (Invitrogen) using the provided universal primers and gene-specific forward primer F2 (5′-ATCGATCGACGTCTACCTG-3′) and reverse primer R5 (5′- CAGAGTAGCCCTAGGAGTAACCACT-3′). Amplification was carried out according to the manufacturer's instructions, and cDNA-RACE products were sequenced as described previously. All *D. v. virgifera* GABA receptor sequences derived from RACE products were aligned using NTI vector (Invitrogen) and consensus sequences to query the NCBI nr protein database and the BLASTX algorithm for annotation and protein domain identification.

Bacterial artificial chromosome library screening and sequencing

The PCR primers RdlPCR for SNP F1 (5′-ATG GAG ATC AGC CTT ACG ACA GG-3′) and RdlPCR for SNP R3 (5′-CAT GGT CAA CAC AGT GGT TAC TCC-3′) were used to screen a *D. v. virgifera* BAC library by PCR as described by Coates *et al*. (2012). Putative positive DvvBAC library clones were cultured in lysogeny broth media containing 15 mg/l chloramphenicol, and BAC plasmid DNA was purified using Qiagen Large Construct Kits (Qiagen) according to the manufacturer's instructions. Approximately 0.5 μg of BAC DNA was digested with 1 unit of *Eco*R1 or *Pst*I (New England Biolabs, Beverly, MA, USA) in single reactions, and in a double digestion reaction. Restriction enzyme reactions also included 3 μl of 10X buffer in a 30 μl reaction that was incubated at 37 °C for 4 h. Digestion products were separated on 25 cm 0.9% agarose gels in 1X TAE for 6 h at 90V, with lambda *Eco*R1 + *Hind*III standard used for size comparison.

Three mid-tagged libraries, RL6 to RL8, were created from three individual BAC DNAs, pooled and sequenced on a one-quarter gasketed Roche GS-FLX Titanium plate at the William H. Keck Center for Comparative and Functional Genomics at the University of Illinois. Raw sequence data were trimmed to remove adapters and low-quality sequence, and subsequent assembly steps were carried out as described previously by Coates *et al*. (2012). VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) was used to identify cloning vector sequence, and was masked using Maskseq (Rice *et al*., 2000). Contigs from all clones were assembled into a consensus reference using CAP3 (Huang & Madan, 1999) (default parameters). A local instance of the NCBI nr protein database was created in a BLAST database, and queried with resultant DvvBAC contigs using the BLASTX algorithm (Altschul *et al*., 1990; *E*-value cutoff $\leq 10^{-10}$). Similarly, the DvvBAC contigs were used to create a local BLAST database, and queried with the DvvGABA receptor cDNA sequence (obtained earlier) using the BLASTN algorithm (*E*-value cutoff $\leq 10^{-50}$). Sequence tracks were annotated using the Artemis Genome Browser and Annotation Tool (Rutherford *et al*., 2000).

Correlation of the Rdl *T^{838} single nucleotide polymorphism with cyclodiene resistance*

The *D. v. virgifera Rdl* mutation (SNP G/T^{838}) was detected using a GenomeLab SNPStart Primer Extension Kit (Beckman Coulter). PCR amplification of the gene region containing the G/T^{838} SNP used the primer pair *Rdl* PCR for SNP F1 and *Rdl* PCR for SNP R, with cDNA template from the susceptible non-diapause strain ($n = 20$) and from field collected beetles from Nebraska ($n = 18$), Illinois ($n = 18$) and Pennsylvania ($n = 23$). PCR amplifications were carried out using 1.5 units of *Taq* polymerase, 0.25 μl dNTP (10 mM), and 1 μl of the 5′ and 3′ primers (each at 10 μM) in 1X *Taq* polymerase buffer (50 mM KCl/10 mM Tris*HC1, pH 8.3/2.5 mM $MgCl_2$, 0.01% gelatin; New England Biolabs) in 10 μl reaction volume. The DNA was amplified by denaturation at 98 °C for 3 min, then by 38 cycles of annealing at 61 °C for 25 s, extension at 72 °C for 25 s, and denaturation at 94 °C for 15 s, and a final extension at 72 °C for 5 min. The internal interrogation primer *Rdl* F2 (5′-TGA ATC GTA ACG CAA CCC CCG CCA GAG TA-3′) was designed with either the GCC to TCC nucleic acid prior to the point mutation at the 3′ end and used in the SNP extension with the PCR products as a template according to the manufacturer's instructions. The primer extension products and SNP genotyping was carried out on a CEQ 8000 Genetic Analysis System and characterized with fragment analysis software in the Genomics Laboratory, USDA-ARS, Lincoln, NE, USA. The correlation between the frequency of the T (putative resistance) allele and mortality in the aldrin bioassay was analyzed by linear regression.

Alternatively, the *Rdl* mutation in genomic DNA was detected by restriction endonuclease digestion of PCR-amplified products (PCR-RFLP). Specifically, the PCR primer pair GABA_SNP-F (5′-TCT GGT TGA ATC GTA ACG CAA C-3′) and GABA-SNP-R (5′-ACG AAG CAA GTC CCC AGG TA-3′) were designed using Primer3 (Rozen & Skaletsky, 2000) using default parameters, except min T_m was increased to 60 °C with the resulting amplified region inclusive of the G/T^{838} SNP (*Rdl* mutation). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA) and 2.5 pmol of each used in 10 μl reactions with 2.5 mM $MgCl_2$, 150 μM dNTPs, 20 ng DNA, primer, 2 μl 5X PCR buffer and 0.3125U Go*Taq* DNA polymerase (Promega, Madison, WI, USA). Amplification took place on a Tetrad2 thermocycler (Bio-Rad, Hercules, CA, USA) using the program TD2 (Coates *et al*., 2009). The PCR products were digested with 0.5 units of the restriction enzyme *Cvi*KI-1 (New England Biolabs) in 2 μl 10X reaction buffer and 8 μl nuclease free water, incubated at 37 °C. Electrophoresis on 1 mm × 20 cm 6% polyacrylamide gels, staining and visualization was conducted as described by Coates *et al*. (2009).

Adult *D. v. virgifera* ($n = 87$) were collected from the Uthe Farm at Iowa State University, Ames, IA, USA in July 2012, and all beetles were exposed to the diagnostic concentration of Aldrin for 24 h as described previously, except for 16 unexposed controls (acetone only). DNA was extracted from individual beetles using

the DNeasy Blood and Tissue kit (Qiagen), quantified on a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and diluted to 10 ng/µl with nuclease-free water and analysed for the *Rdl* mutation by PCR-RFLP. The Pearson product-moment correlation coefficient (PMCC) was estimated between phenotype (unaffected = 1; dead = 3; lethargic = 2) and genotype (number of resistant T^{838} alleles) for paired data with SAS v. 9.2. The significance of the correlation (r) was estimated assuming the population of the true correlations (θ) with size N was distributed approximately as t, where $t = r \times [(n-2)/(1-r^2)]^{0.5}$.

Distribution of the Rdl T^{838} *allele in North America*

DNA samples previously prepared from beetles collected at New Deal, DE, Ankeny, IA, Concordia, KS, Mead, NE, Cobleskill, NY and Bowersville, OH, USA (Kim & Sappington, 2005b; Kim *et al.*, 2008) were genotyped using the *Cvi*KI-1 PCR-RFLP assay. The significance of deviation between H_O and H_E were estimated using Markov chain exact tests using the ARLEQUIN software package (v. 3.5.1.2; Excoffier & Lischer, 2010), and significance of deviation from Hardy-Weinberg Equilibrium was assessed with an $\alpha = 0.05$. Pairwise F_{ST} estimates were also calculated using ARLEQUIN (v. 3.5.1.2; Excoffier & Lischer, 2010), and significant differences among sample sites were determined after correcting for inflated experiment-wise Type I error rates incurred during multiple hypothesis tests using the B-Y method $\alpha/(1 \div 1) + (1 \div 2) + (1 \div 3) + \ldots (1 \div k)$, where k is the number of hypothesis tests performed (Benjamini & Yekutieli, 2001). Population structure was evaluated by AMOVA using ARLEQUIN 3.5.1.2. Isolation-by-distance was examined by regressing $F_{ST}/(1 - F_{ST})$ on $\log_{10}$ geographic distance between sample sites using the IBD web service v.3.15 (http://ibdws.sdsu.edu/~ibdws/; Jensen *et al.*, 2005), and significance was estimated by Mantel Tests with 1000 jackknife permutation steps.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Alignment of the deduced amino acid sequence of the *Diabrotica virgifera virgifera* gamma-aminobutyric acid (GABA) receptor gene from the susceptible non-diapause strain with GABA receptor isoform b from *Tribolium castaneum*. An asterisk above sequence indicates position of the alanine (A) to Serine/Glycine (S/G) substitution observed in cyclodiene-resistant species (ffrench-Constant *et al*., 2000). Transmembrane domains (TM1-TM3) are boxed.

Figure S2. Restriction fragment length polymorphism of *D. v. virgifera* BAC clones that encode the gamma-aminobutyric acid (GABA) receptor marker. L = Lambda *Eco*RI + *Hind*III ladder; lane 1: clone 47B13, lane 2: clone 82A13, lane 3: clone 129I06, lane 4 clone 178L17, lane 5, clone 222A09; lane 6 clone 236O22; lane 7, clone 248K09; lane 8, clone 269I11; lane 9, clone 274K02.

Table S1. BLASTX search results of the National Center for Biotechnology Information nonredundant protein database queried by the *Diabrotica virgifera virgifera* gamma-aminobutyric acid (GABA) receptor gene. Percent identities are to the deduced *D. v. virgifera* amino acid sequence.

Supplemental Data

Table S1. BLASTx search results of the NCBI nr protein database queried by the *D. v. virgifera* GABA receptor gene. Percent identities are to the deduced *D. v. virgifera* amino acid sequence.

Species	Protein Sequence Identity	% Identity
Drosophila melanogaster	304/326	93
Oulema oryzae	355/391	91
Tribolium castaneum	354/399	89
Plutella xylostella	322/366	88
Heliothis virescens	319/365	87
Apis mellifera	316/364	87
Anopheles gambiae	342/472	72
Lucilia cuprina	324/377	86
Musca domestica	323/407	79

Figure S1: Alignment of the deduced amino acid sequence of the *D. v. virgifera* GABA receptor gene from the susceptible non-diapause strain with GABA receptor isoform b from *Tribolium castaneum*. * above sequence indicates position of the alanine (A) to Serine/Glycine (S/G) substitution observed in cyclodiene-resistant species (ffrench-Constant *et al*. 2000). Transmembrane domains (TM1-TM3) are boxed.

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T.castaneum  MGHSRVVWPAVLLALALP-WASAGSPGAGGSYLGDVNISAILDSFSVSYDKRVRPNYGGP 59
D.v.v.       MGRSRMVWPAVLLTLVAFGGAFAGSPGAGGSYVGGVNVSAILDSFSVSYDKRVRPNYGGP 60
             **:**:*******:*.    * **********:*.**:**********************

T.castaneum  PVEVGVTMYVLSISSLSEVKMDFTLDFYFRQFWTDPRLAFRKRPGVETLSVGSEFIKNIW 119
D.v.v.       PVDVGVTMYVLSISSLSEVQMDFTLDFYFRQFWTDPRLAFRKRPSVEILSVGSEFIKNIW 120
             **:****************:************************.** ************

T.castaneum  VPDTFFVNEKQSYFHIATTSNEFIRIHHSGSITRSIRLTITASCPMNLQYFPMDRQLCHI 179
D.v.v.       VPDTFFVNEKHSSFHMATTSNEFIRIHHSGSITRSIRLTITASCPMNLQYFPMDRQVCHI 180
             **********:* **:****************************************:***

T.castaneum  EIESFGYTMRDIRYKWNEGPNSVGVSNEVSLPQFKVLGHRQRAMEISLTTGNYSRLACEI 239
D.v.v.       EIESFGYTMRDIRYKWNEGPNSVGVSNEVSLPQFKVLGHRQRAMEISLTTGNYSRLACEI 240
             ************************************************************
                              TM1                 *       TM2
T.castaneum  QFVRSMGYYLIQIYIPSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTLMSSTNAAL 299
D.v.v.       QFVRSMGYYLIQIYIPSGLIVIISWVSFWLNRNATPARVALGVTTVLTMTTLMSSTNAAL 300
             **************************************************************
                          TM3
T.castaneum  PKISYVKSIDVYLGTCFVMVFASLLEYATVGYMAKRIQMRKNRFLAIQKIAEQKKLNVDG 359
D.v.v.       PKISYVKSIDVYLGTCFVMVFASLLEYATVGYMAKRIQMQKNRFLAIQKIAEQKKLNVDG 360
             ********************************************:*******************

T.castaneum  GPDSDHAPKQTVSRPIGHEVRFKVHDPKAHSKGGTLESTVNGGRGGGGGGGPDEEAAAPI 419
D.v.v.       GPD-DHAPKQTV--------RFKVHDPKAHSKGGTLESTRKRRSVGVRQRG--------- 402
             *** ********        ******************* :    *    *

T.castaneum  PQHIIHPNKDINKLYGITPSDIDKYSRIVFPVCFVCFNLMYWIIYLHISDVVADDLVLLE 479
D.v.v.       ---------------------------------CYTH------------------------ 406
                                              *:.

T.castaneum  EDK 482
D.v.v.       ---
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Figure S2: Restriction Fragment Length Polymorphism (RFLP) of *D. v. virgifera* BAC clones that encode the gamma-aminobutyric acid (GABA) receptor marker. L = Lambda *Eco*RI + *Hind*III ladder; lane 1: clone 47B13, lane 2: clone 82A13, lane 3: clone 129I06, lane 4 clone 178L17, lane 5, clone 222A09; lane 6 clone 236O22; lane 7, clone 248K09; lane 8, clone 269I11; lane 9, clone 274K02