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Genetic variation in geographical populations of western and Mexican corn rootworm

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Abstract

Genetic variation in the nuclear rDNA ITS1 region of western corn rootworm, *Diabrotica virgifera virgifera* (WCR), and Mexican corn rootworm, *D. v. zeae* (MCR) was studied. Two sites were detected which differentiated WCR and MCR in the 642-base sequence. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of the first internal transcribed spacer region (ITS1) sequence revealed no variation within or among the twelve WCR and two MCR populations. PCR-RFLP of 75% of the mitochondrial DNA genome detected one significant polymorphic site out of the approximately 190 restriction sizes observed in WCR. The polymorphism did not differentiate geographical populations of WCR and is not diagnostic for the subspecies. The low levels of variation observed in WCR suggests either high levels of gene flow or a recent geographical expansion from a relatively small base. Gene flow would facilitate the rapid spread of traits that could compromise control programmes, such as insecticide resistance or behavioural modifications. The minimal genetic differentiation between WCR and MCR raises questions about the evolutionary history of these subspecies and how the distinct phenotypes are maintained.

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Introduction

Western corn rootworm, *Diabrotica virgifera virgifera* LeConte (WCR), and Mexican corn rootworm, *D. v. zeae* Krysan & Smith (MCR) along with several other species of diabroticite beetles are among the most economically and environmentally important insect pests of maize production systems in the USA (Levine & Oloumi-Sadeghi, 1991). Diabroticite beetles are members of Chrysomelidae, many of which attack agricultural crops. Other major pests in the group include Northern corn rootworm, *Diabrotica barberi* Smith and Lawrence (NCR), western spotted cucumber beetle, *D. undecimpunctata undecimpunctata* Mannerheim (WSCB) and southern corn rootworm, *D. u. howardi* Barber (SCR). In the USA, 20–25 million acres of corn are treated annually with soil insecticides to protect the crop from corn rootworm larval feeding damage (Fuller *et al*., 1997). Soil insecticides applied for corn rootworm represent one of the major uses of insecticide in the USA. In some locations, insecticides are used against adult beetles as well. This is costly both economically and environmentally. The economic impact of WCR has prompted interest in an area-wide management approach using integrated pest management (IPM) procedures to control rootworm damage.

WCR is distributed from north-western Mexico through the central plains of the USA and east to New Jersey, excluding the south-eastern USA. In the south-western USA, WCR is replaced by MCR which ranges from central America through eastern Mexico to Texas and Oklahoma (Krysan, 1986; Krysan & Smith, 1987). MCR has been observed infesting sorghum as well as corn (Stewart *et al*., 1995). WCR has spread rapidly eastward across the USA from the Western Great Plains to the east coast since 1940. Some populations of WCR have developed insecticide resistance in a relatively short period of time (Ball & Weekman, 1963; Metcalf, 1983; Meinke *et al*., 1998). Other populations have shown a propensity to disrupt

crop rotation programmes by depositing eggs outside of corn fields, particularly in soybeans (Levine & Oloumi-Sadeghi, 1991). Morphological features can distinguish adult MCR and WCR except in a narrow zone of overlap where a few intermediate phenotypes have been observed. However, larvae cannot be differentiated (Krysan *et al.*, 1980; Krysan, 1986). Identification of *Diabrotica* larvae to species is difficult and for several species is impossible (Krysan, 1986). Despite the benefits that genetic analyses of WCR and MCR may provide towards diagnostics, dispersal, insecticide resistance and the implementation of area-wide control programmes, little research has been conducted on these two important subspecies.

The purpose of this study was to examine genetic variation in local and dispersed populations of WCR and MCR. Portions of the nuclear and mitochondrial genomes were examined. The first internal transcribed spacer region (ITS1), located between the 18S and 5.8S nuclear ribosomal DNA (rDNA) genes, was amplified using polymerase chain reaction (PCR) and subjected to DNA sequencing and restriction fragment length polymorphism (RFLP) analyses. PCR and long PCR was used to amplify two amplicons, representing about 75% of the mitochondrial DNA (mtDNA) for RFLP analysis.

Results

The ITS1 primer pair produced amplicons of 642 bp for both WCR and MCR. Three WCR (Brookings, SD; Dey, NE; Mead, NE) (GenBank accession numbers AF155569 to AF155571), and two MCR (Temple, TX; Uvalde, TX) (GenBank AF155572, AF155573), were sequenced (Table 1). No sequence variation was detected in the amplified portions of the 18S and 5.8S ribosomal RNA (RNA) coding regions among WCR and MCR. No intrasubspecific variation was observed within either WCR or MCR, but two ITS1 sites differentiated the subspecies. Nucleotide 367 of MCR is 'G' whereas WCR has a 'T' at this position. At nucleotide 598, a poly A run is 9 bp in MCR, but only 8 in WCR (Fig. 1). Sequence divergence between WCR and MCR is about 0.3% in the ITS1 region. The ITS1 region of SCR was also amplified and sequenced (GenBank U90334) and NCR (GenBank AF155574) for comparison (Fig. 1). The SCR sequence was 752 bp, approximately 110 bp larger than WCR, MCR and NCR. A 102-bp insert between the 3′ end of the 18S gene and ITS1 and a 14-bp insertion within ITS1 accounted for the size difference. Nucleotide sequence divergence between *D. virgifera* and SCR was about 13% when the two large insertions are considered as single traits.

PCR-RFLP analysis of the ITS1 amplicon using the restriction enzymes *Apo*I, *Dde*I, *Dra*I, *Rsa*I and *Taq*I detected fourteen restriction sites (Table 2). Ten of the fourteen restriction sites were in the 417-bp ITS1 region

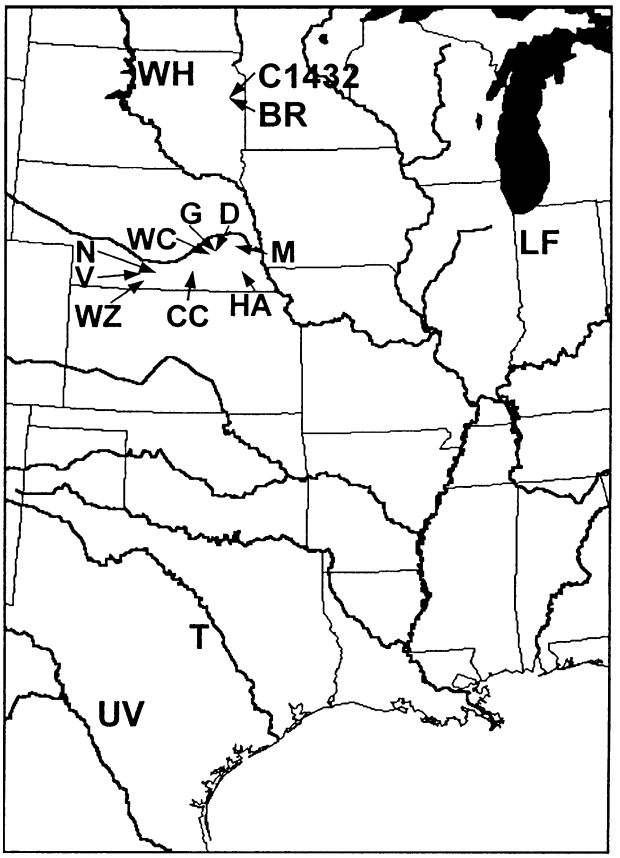

Figure 1. Western and Mexican corn rootworm collection sites. Abbreviations correspond to Table 1.

and screened approximately 12% of the region. Analysis of four to five individuals per population revealed no variation within or among twelve WCR populations (LF excluded) and two MCR populations. None of the restriction enzymes in the Webcutter database (Heiman, 1997) differentially cleaved either of the polymorphic sites observed in the nucleotide sequences.

The mtDNA long PCR amplicons 12S-N4 (5.7 kb) and CB2H-C1 (8.7 kb) partially overlap and together represented about 12 kb of the mitochondrial genome. *Alu*I, *Ase*I, *Dpn*II, *Dra*I, *Hin*fI, *Ssp*I, *Swa*I and *Xba*I were used for the RFLP analysis. Seventy-one individuals from twelve of the thirteen WCR collections (HA excluded) and forty-six MCR from the two locations were analysed. Cleaving the PCR amplicons C1–C2 (1.6 kb) and 12S–16S (1.2 kb) with some of the aforementioned restriction enzymes and *Mse*I revealed at least twenty additional restriction sites (data not shown). The approximately 190 restriction fragments detected corresponded to about 170 restriction sites (Table 2). The number of mtDNA base pairs tested by the battery of restriction enzymes exceeds 900. Only one major polymorphism was observed in WCR, a 510-bp fragment in the *Dpn*II digests of the 12S-N4 fragment (Fig. 2). The band was present (pattern

Table 1. Sampling locations and dates for collections of *Diabrotica* spp.

Population	Species	Sampling Location (city, county, state)	Resistance Status[a]	Date
CC	WCR	Clay Center, Clay Co., NE	S	1996
HA	WCR	Havelock, Lancaster Co., NE	S	1994
N	WCR	Nichie, Phelps Co., NE	R	1996
WZ	WCR	Winz, Phelps Co., NE	R	1996
V	WCR	Vandell, Phelps Co., NE	R	1996
M	WCR	Mead, Saunders Co., NE	S	1996
G	WCR	Gresham, York Co., NE	R	1996
WC	WCR	Waco, York Co., NE	S	1996
D	WCR	Dey, York Co., NE	R	1996
C1432	WCR	Brookings, Brookings Co., SD	S	1996
BR	WCR	Brookings non-diapause colony	S	1996
WH	WCR	Whitlock Bay, Potter Co., SD	S	1996
LF	WCR	West Lafayette, Tippecanoe Co., IN[b]	S	1997
T	MCR	Temple, Bell Co., TX	S	1996
UV	MCR	Uvalde, Uvalde Co., TX	S	1997

[a]Meinke *et al.*, 1998. Resistant (R) or Sensitive (S) to methyl parathion and carbaryl.
[b]Insects were obtained from a colony maintained by French Agricultural Research, Lamberton, MN. The original collection site and date collected are unknown.

Table 2. Western (WCR) and Mexican corn rootworm (MCR) restriction fragment patterns from ITS1 and mitochondrial DNA amplicons.

Amplicon	Enzyme		Subspecies[a]	Estimated fragment size (bp)
ITS1	*Apo*I		WCR/MCR	421, 158, 63
	*Dde*I		WCR/MCR	306, 261, 75
	*Dra*I		WCR/MCR	479, 99, 36, 28
	*Rsa*I		WCR/MCR	227, 198, 108, 86, 23
	*Taq*I		WCR/MCR	277, 132, 101, 75, 57
mtDNA				
12S-N4	*Hin*fI		WCR/MCR	1550, 1500, 670, 600, 450, 440, 170, 130
	*Swa*I		WCR/MCR	~3200, 800, 750, 350, 310 (170)
	*Hae*III		WCR/MCR	1900, 1500, 1300, 750
	*Alu*I	'A'	WCR (67)	1500, 1050, 490, 485, 395, 370, 310, 297, 280, 240, 195, 175, 75*, 60*
			MCR (46)	"
		'B'	WCR (3)	1500, 1050, 490, 485, 395, 370, 310, 297, 280, 240, 195, 180, 175
		'C'	WCR (1)	1500, 1050, 490, 485, 395, 370, 310, 297, 240, 195
	*Dpn*II	'A'	WCR (47)	1350, 1250, 590, 470, 310, 240, 220, 170
			MCR (46)	"
		'B'	WCR (24)	1350, 1250, 590, 510, 470, 310, 240, 220, 170
	*Ssp*I		WCR/MCR	850, 750, 600, 315, 240, 225, 200, 195, 180, 177, 140, 135, 110, 100, 95, 90, 85, 80, 75
	*Ase*I		WCR/MCR	900, 850, 390, 355, 335, 230, 195, 190, 180, 170, 160, 120–90 (6 band pattern), 70, 65
	*Dra*I		WCR/MCR	1100, 950, 360, 310, 265, 240, 235, 180, 170, 140, 90, 80, 75, + 7* bands < 60
CB2H-C1	*Hin*fI		WCR/MCR	3600, 1750, 1400, 1350
	*Swa*I		WCR/MCR	3100, 1600, 1500, 810, 750, 510, 355
	*Alu*I		WCR/MCR	1500, 720, 700, 495, 490, 410, 405, 395, 360, 345, 315, 295, 275, 250, 235, 195, 170, 150, 135, 110, 90, 75, 65
	*Dpn*II		WCR/MCR	3200, 1350, 1250, 710, 380, 285, 260, 220, 130
	*Ssp*I	'A'	WCR (69)	1000, 700, 680, 350, 345, 280, 260, 220, 205, 195, 185, 175, 160, 140, 130, 120, 105, 100
			MCR (46)	"
		'B'	WCR (1)	1000, 700, 680, 410, 350, 280, 260, 220, 205, 195, 185, 175, 160, 140, 130, 120, 105, 100
		'C'	WCR (2)	850, 700, 680, 350, 345, 280, 260, 220, 205, 195, 185, 175, 160, 140, 130, 120, 105, 100
	*Ase*I		WCR/MCR	800, 750, 650, 600, 400, 385, 365, 350, 310, 300, 285, 240, 239, 220, 200, 195, 190, 185, 180, 170, 160, 150, 120, 100, 95, 90, 80, 70, 60, 50, 40
	*Dra*I		WCR/MCR	1900, 750, 550, 507, 450, 360, 350, 340, 300, 290, 280, 270, 240, 235, 215, 200, 190, 185, + 8 bands < 120
16S2-C2R	*Xba*I		WCR (37)	6300, 1850, 600, 495
			MCR (6)	"
C1-C2	*Mse*I		WCR (8)	200, 165, 155, 110, 95, 87, 65, 50, 42
			MCR (6)	"

[a]WCR ITS1, 45 individuals, MCR ITS1 6 individuals; WCR 12S-N4, 71 individuals; WCR CB2H-C1, 72 individuals; MCR 46 individuals; () indicates the number of individuals with polymorphic pattern.
*Bands that were not visible on all gels.

```
     1                                                  50
MCR  TTGATTACGT CCCTGCCCTT TGTACACACC GCCCGTCGCT ACTACCGATT
WCR  .......... .......... .......... .......... ..........
NCR  .......... .......... .......... .......... ..........
SCR  .......... .......... .......... .......... ..........
     51                                                100
MCR  GAATGATTTA GTGAGGTCTT CGGACTGAGC GCGGTGGCGT TTCGGCGTCG
WCR  .......... .......... .......... .......... ..........
NCR  .......... .......... .......... .......... ..........
SCR  .......... .......... .......... .......... ..........
     101                                               150
MCR  TCGATGTTTC GGAAAGATGA CCAAACTTGA TCATTTAGAG GAAGTAAAAG
WCR  .......... .......... .......... .......... ..........
NCR  .......... .......... .......... .......... ..........
SCR  .......... .......... .......... .......... ....G.C...
     151                                               200
MCR  TCGTAACAAG GTTTCCGTAG GTGAACCTGC GGAAGGATCA TTACAGTGTT
WCR  .......... .......... .......... .......... ..........
NCR  .......... .......... .......... .......... ..........
SCR  .......... .......... .......... .......G.. ..........
     201                                               250
MCR  GTCAATAAAC GACAAGTCTG TACTAAATTA TTTCGAACTG TTAAAATAAC
WCR  .......... .......... .......... .......... ..........
NCR  .......... .......... .......... .......... ..........
SCR  .......... .......... .......... .......... ..........
     251                                               300
MCR  TGAC------ ---------- ---------- ---------- ----------
WCR  ....------ ---------- ---------- ---------- ----------
NCR  ....------ ---------- ---------- ---------- ----------
SCR  ....GTGTGT CTTATCTTCG TATACTGCAA GGAACAAAAA TTAATAGTAA
     301                                               350
MCR  ---------- ---------- ---------- ---------- ----------
WCR  ---------- ---------- ---------- ---------- ----------
NCR  ---------- ---------- ---------- ---------- ----------
SCR  TCAATTATGC TTTATTGTGG AAAATACGAA AAAATAGAGA CACATACACG
     351                                               400
MCR  ------GTTA TTATCTGTGT GATC--GTAG AATAAAATGT TTTGATATAA
WCR  ------.... ......T... ....--.... .......... ..........
NCR  ------.... ......T... ....--.... .......... ..........
SCR  TGTATT.... ....TAT... ....GT.... .TGC...ACG ...T.ATGT.
```

```
     401                                                 4
MCR  CAAAATATCT CTCGACAATA TATTATATCG GGTACCTAAA ATATTTCGG-
WCR  .......... .......... .......... .......... .........-
NCR  .......... ..A....... .......... ........G. .........-
SCR  AT........ .GATAAT... .CGGG..CCT A.AGAACG.. G.C......G
     451                                                 5
MCR  ---------- ---TGGTGAT AAAGATTTTC GCCCGATTGT CAAGAGATCT
WCR  ---------- ---....... .......... .......... ..........
NCR  ---------- ---....... .......... .......... ..........
SCR  ATTAGTTCTT CGA....... .......... ........A. ........G.
     501                                                 5
MCR  TAGACTTAAA CGTAATTTCT TGATCGCTTT AAGAGAGGAA ATTCGTGTAT
WCR  .......... .......... .......... .......... ..........
NCR  .......... .......... .....A.... .......... ...T.....A
SCR  ..C...G... .......... .......A.. .......... .........C
     551                                                 6
MCR  AGTTTTTTGG CACACCTCGT TTGCTAAACA TGTGTGTTGT GCTATTTAAA
WCR  .......... .......... .......... .......... .......-..
NCR  .......... .......... .....T.... .......... .......-..
SCR  C.......-. ...G...... ...T.T.... .A....C... ..--..A...
     601                                                 6
MCR  AAAAAACCTA ATATAC---C TCCTTTTTTA ATTTGGCTAA ATATACTTAT
WCR  .......... ......---. .......... .......... ..........
NCR  .......... .A....AGTT .......... ......A... ..........
SCR  .......G.. ...A..-GAT .T.....C.G ...C.TA... .......CGC
     651                                                 7
MCR  CATTTTACAT AAAAGACGTT GT-TTTCGTA CTTAAATAAA CTTTTATGTT
WCR  .......... .......... ..-....... .......... ..........
NCR  .......... .......... ..G....... .......... ..........
SCR  ......CT.. ...T...... ..G....... .......... .....G....
     701                                                 7
MCR  TAAATTTTCG AACGGAACGC GTCTAATTTA AACGATTACC CTGAACGGTG
WCR  .......... .......... .......... .......... ..........
NCR  .......... .......... .......... .......... ..........
SCR  AT........ .......... ......-... .......... ..........
     751                765
MCR  GATCACTCGG CTCGT
WCR  .......... ....
NCR  .......... ....
SCR  .......... ....
```

Figure 2. Sequence comparison for *D.* vs. *vigifera* (WCR), *D.* vs. *zeae* (MCR), *D. barberi* (NCR), and *D. undecimpunctata howardi* (SCR). '.' indicate that the same nucleotide is present as that in MCR; -, indicates a deletion. Primer sequences are underlined and encoding regions (18S and 5.8) are italicized. GenBank accession numbers are (MCR), (WCR), (NCR), and U90334 (SCR).

'B') in 34% (24/71) of the individuals. Both patterns were observed in each of the twelve collections examined (Table 3) and therefore are not useful for recognizing geographical nor insecticide resistance populations. All MCR had *Dpn*II pattern 'A'. Four other rare polymorphisms were observed. 12S-N4 *Alu*I 'B' was found in three individuals from the Brookings colony; 12S-N4 *Alu*I 'C' was found in a Clay Center individual; CB2H-C1 *Ssp*I 'B' was found in one Gresham individual; and CB2H-C1 *Ssp*I 'C' was found at Gresham and Clay Center (Table 2). RFLP analysis of mtDNA could not differentiate MCR from WCR (Fig. 3).

Discussion

The low level of genetic differentiation observed within and among WCR and MCR populations is supported by one allozyme study. A phylogenetic study of five *Diabrotica* species (Krysan *et al.*, 1989) found minimal genetic differentiation among fifteen WCR and six MCR populations. Among other diabroticite beetles, low levels of allozyme

Table 3. Distribution of mtDNA 12S-N4 *Dpn*II restriction fragment patterns 'A' and 'B'.

Population	R/S[a]	'A' individuals	'B' individuals
WCR			
CC	S	4	1
N	R	3	1
WZ	R	4	2
V	R	3	3
M	S	2	2
G	R	4	2
WC (NE)	S	5	1
D	R	3	1
C1432	S	1	3
BR	S	4	5
WH	S	4	2
LF	S	10	1
total WCR		47 (66%)	24 (34%)
MCR			
T	S	20	0
UV	S	26	0
Total MCR		46 (100%)	0

[a]Resistant (R) or Sensitive (S) to methyl parathion and carbaryl.

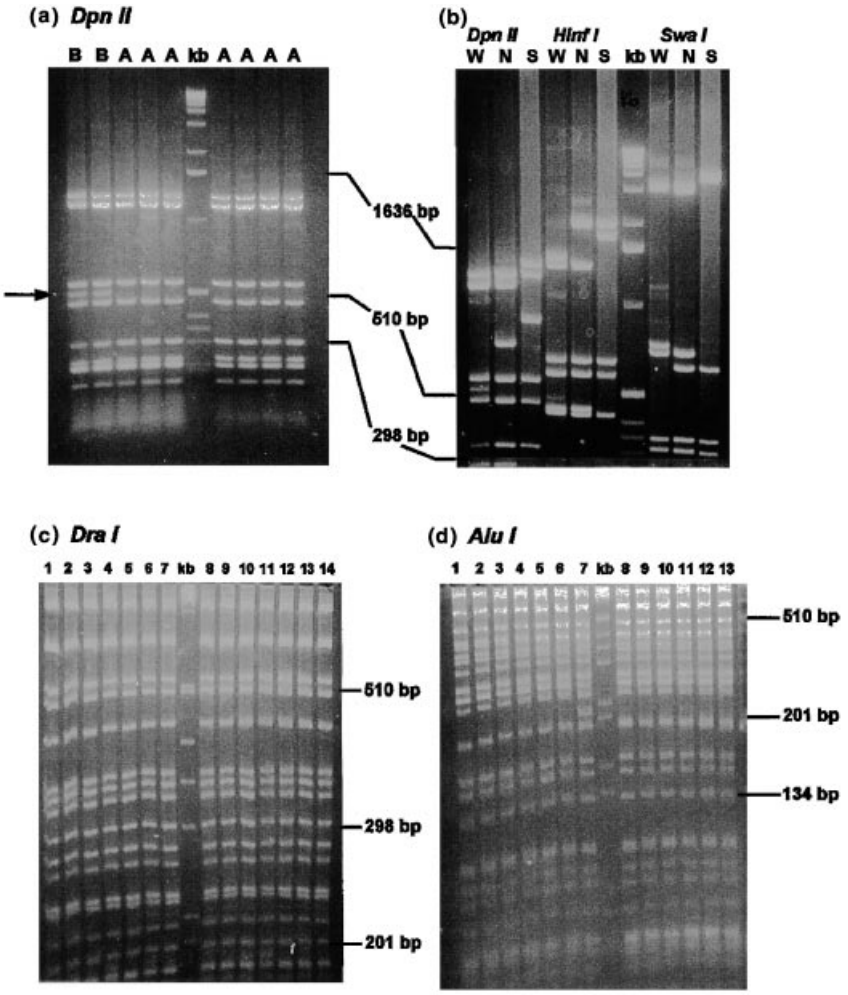

Figure 3. PCR-RFLP of corn rootworm mtDNA. (a) *Dpn*II fragments from WCR 12S-N4 amplicon showing 'A' and 'B' restriction patterns. Arrow denotes extra fragment in 'B' pattern. Lane 1, C1432; lanes 2–3, N; lanes 4–5; D; lane 6, kb ladder; lane 7, UV; lanes 8–10, BR. (b) 12S-N4 amplicon from WCR, NCR and SCR. Lanes 1–3, *Dpn*II; lanes 4–6, *Hin*fI; lane 7, kb ladder DNA marker; lanes 8–10, *Swa*I. (c) *Dra*I fragments from WCR CB2-C1 amplicon. Lanes 1–2, C1432; lanes 3–4, N; lanes 5–6, D; lanes 7–8, M; lanes 9–11, BR; lanes 12–14 WZ. (d) *Alu*I fragments from WCR CB2-C1 amplicon. Lane 1, WZ; lanes 2–5, G; lanes 6–8, CC; lanes 9–10, V; lanes 11–12, WC; lane 13, WH. 1 kb ladder used as DNA size marker on all gels. Band size labels refer to the corresponding band in the marker lane.

variation have also been observed in NCR. McDonald *et al.* (1985) did not detect any significant genetic differentiation among thirty-three geographically isolated populations of NCR. In a similar study, Krafsur *et al.* (1993) analysed twenty-one polymorphic loci from fourteen populations of NCR and showed high levels of gene flow among populations. Krafsur (1995) examined univoltine and semivoltine NCR populations and concluded that gene flow was virtually unrestricted between the phenotypes. However, significant ITS1 and mtDNA variation has been observed in NCR, and appears to be substantially greater than that within WCR (A. Szalanski and R. Roehrdanz, unpublished data).

Because only three WCR and two MCR were sequenced, we remain very cautious about claiming that the two site polymorphisms are diagnostic. Low levels of genetic diversity in the ITS1 and mtDNA regions among widely dispersed populations have been reported for other insect species including European corn borer, *Ostrinia nubilalis* (Marcon *et al.*, 1999), stable fly, *Stomoxys calcitrans* (Szalanski *et al.*, 1996), tobacco budworm, *Heliothis virescens* (Roehrdanz *et al.*, 1994), monarch butterfly, *Danaus plexippus* (Brower & Boyce, 1991), gypsy moth, *Lymanatria dispar* (Bogdanowicz *et al.*, 1993) and alfalfa weevil, *Hypera postica* (Erney *et al.*, 1996). These species have high mobility or recent expansion from a genetic bottleneck in common. Gene flow is enhanced by high mobility as in tobacco budworm and monarch. Recent expansion is favoured by introduction into new habitats as for European corn borer, gypsy moth and alfalfa weevil. These factors may be contributing to the lack of genetic variation among WCR populations as well.

The absence of mtDNA variation between the WCR and MCR subspecies indicates a recent common evolutionary history. The boundary between the two phenotypes has remained discrete and stationary, even as WCR expanded eastward. Presumptive hybrid beetles have been collected infrequently along a narrow boundary between the two populations in the panhandle of Texas and central Mexico (Krysan *et al.*, 1980; Giordano *et al.*, 1997). Laboratory crosses between WCR and MCR displayed reduced egg hatch when WCR was the paternal parent

(Krysan & Branson, 1977). Electron microscopy revealed rickettsia-like bacteria, later classified as *Wolbachia*, in the reproductive tract of male WCR but absent in MCR (Degrugillier *et al.*, 1991; Giordano *et al.*, 1997). These *Wolbachia* appear to be responsible for the observed cytoplasmic incompatibility and may be a primary factor in maintaining the boundary between WCR and MCR. WCR cured of *Wolbachia* with tetracycline are fertile when crossed with MCR (Giordano *et al.*, 1997). Because ITS1 and mtDNA PCR-RFLPs cannot differentiate between WCR and MCR, analysis of intermediates will not resolve the origin.

The paucity of genetic polymorphism in the ribosomal spacer and mitochondrial DNA diminishes the prospect of using these tools to quickly find molecular markers to track insecticide resistance, expansion of host range to include sorghum, increased tendency to deposit eggs outside of corn fields, or other behavioural modifications. Techniques detecting higher levels of polymorphism such as RAPD-PCR or AFLP may reveal markers more suitable for population level analyses of altered behaviours and insecticide resistance in WCR. Assays for genes or gene products directly involved may also prove useful, especially for resistance management.

Experimental procedures

Rootworm samples

WCR and MCR adults were obtained from the field locations listed in Table 1. In most cases, eggs were obtained from field-collected adults and reared to adulthood at the Northern Grain Insects Research Laboratory, Brookings, SD. F_1 adults were frozen and stored at –80 °C. Field collected beetles from Lancaster County, NE and Uvalde, TX, were preserved in 70% ethanol. Populations WZ, N and V from Phelps County, Nebraska, and populations D and G from York County, Nebraska, have been identified as resistant to methyl parathion and carbaryl. Other Nebraska populations are susceptible to those insecticides, and resistance has not been reported outside of Nebraska Meinke *et al.* (1998). Adult rootworms were preserved by immersion in 70% alcohol or freezing at −80°C. Frozen voucher specimens are being maintained at the ARS-USDA Biosciences Research Laboratory, Fargo, ND.

DNA extraction, amplification and sequencing

DNA for ITS1 sequencing was extracted from individual thoraces using a phenol/chloroform technique similar to Taylor *et al.* (1996). The primers rDNA2 (5′-TTGATTACGTCCCTGCCCTTT-3′, Vrain *et al.*, 1992) and rDNA1.58s (3′-GCCACCTAGTGAGCCGAGCA-5′, Cherry *et al.*, 1997) amplified a 3′ portion of the 18S gene, the entire ITS1 region and a 5′ portion of the 5.8S gene. Primers were synthesized by the University of Nebraska-Lincoln primer synthesis lab (Lincoln, NE). ITS1 PCR using 1.0 µL of template DNA was done as described by Szalanski *et al.* (1997). Amplified DNA for sequencing was purified using Geneclean II (Bio 101, Inc. Vista, CA) and sequenced in both directions using a Prism DNA sequencer (Applied Biosystems, Inc.) located at the Iowa State University DNA sequence and synthesis facility (Ames, IA). Consensus sequences were derived from individual sequences in each direction using GAP (Genetics Computer Group, Madison, WI).

DNA for use in long PCR was extracted from individual insects using the high salt procedure of Cheung *et al.* (1993). Long PCR reactions were done as described by Roehrdanz (1995). Primers included C1 (C1-J-2195), C2 (C2-N-3662), N4 (N4-J-8944), 16SR (LR-J-12883), 16S2 (LR-N-12945), 12S (SR-N-14588), CB2H (CB-N-10920) (Simon *et al.*, 1994; Roehrdanz & Degrugillier, 1998). MtDNA primers were synthesized by NBI, Plymouth, MN.

Restriction fragment length polymorphism analysis

Web Cutter 2.0 (Heiman, 1997) was used to predict restriction sites in ITS1 sequence data. ITS1 amplicons were digested according to manufacturer's recommendations (New England Biolabs, Beverly, MA) using the enzymes *Apo*I, *Dde*I, *Dra*I, *Rsa*I and *Taq*I (Cherry *et al.*, 1997). Restriction enzymes *Alu*I, *Ase*I, *Dra*I, *Ssp*I, *Hin*fI, *Swa*I, *Xba*I, *Hae*III, *Scr*F I, and *Dpn*II were used for the mtDNA amplicons. *Mse*I was also used to digest some of the smaller mtDNA amplicons. Digest products were visualized by separation with seakem or metaphor agarose (FMC) or polyacrylamide gel electrophoresis and stained with ethidium bromide (Taylor *et al.*, 1997). The number of base pairs surveyed with each mtDNA amplicon was estimated using the formula (number of restriction fragments – 1) × (length of the recognition sequence for each restriction enzyme). Because there is a 2-kb overlap in the large PCR amplicons the total number of base pairs was corrected for restriction sites counted twice by subtracting the number of bases equal to the ratio of the overlap to the total amplicon length (2 kb/8.7 kb) for one of the amplicons.

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