Wolbachia Bacterial Infections Linked to Mitochondrial DNA Reproductive Isolation Among Populations of Northern Corn Rootworm (Coleoptera: Chrysomelidae)

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ABSTRACT The northern corn rootworm, *Diabrotica barberi* Smith & Lawrence (Coleoptera: Chrysomelidae) is an agricultural pest that ranges from the eastern Dakotas to Kansas and east to the Atlantic coast. The endosymbiotic bacteria *Wolbachia* has been detected in northern corn rootworm populations from east of the Mississippi River. Using the *Wolbachia* 16S rDNA, *fts*Z and *wsp* genes a boundary was identified in central Illinois, between infected and uninfected populations with the infected populations found to the east of the boundary. Sequences of portions of the *Wolbachia fts*Z and *wsp* genes have been obtained from four geographic locations of northern corn rootworm. Within infected northern corn rootworm populations, two strains have been detected. The 1,058-bp *fts*Z sequences from northern corn rootworm indicate that both strains belong to the *Wolbachia* supergroup A. NCR Type 1 *Wolbachia* was found from eastern Illinois to Pennsylvania. NCR Type 2 *Wolbachia* occurs in central Illinois. The ≈600-bp *wsp* sequences from the two strains are also dramatically different. Strain differences in restriction fragment length polymorphism patterns of the *Wolbachia*-specific amplicons were used to determine the distribution of the strains. The boundary between these two strains of *Wolbachia* in native populations of northern corn rootworm correlates with a previously observed mitochondrial DNA genetic boundary in eastern Illinois, suggesting that the two *Wolbachia* strains are incompatible and little if any introgression occurs between the two infected populations. The results demonstrate that *Wolbachia* can influence the genetics of a major insect pest over a wide geographic area and that it may be driving reproductive isolation between populations of northern corn rootworm.

KEY WORDS corn rootworm, *Wolbachia*, mtDNA, *wsp*, *fts*Z

The northern corn rootworm, *Diabrotica barberi* Smith & Lawrence (Coleoptera: Chrysomelidae), is a significant pest of corn, *Zea mays* L., in the north central part of the United States. The range of the insect extends from the eastern Great Plains (North Dakota south to Kansas) eastward to the Atlantic coast (Krysan and Smith 1987). Differences in mitochondrial DNA (mtDNA) suggest an evolutionary history of reproductive isolation between eastern and western populations of northern corn rootworm. An examination of intraspecific geographic variability by using polymerase chain reaction-restriction fragment length polymorphism of long mitochondrial DNA (mtDNA) amplicons revealed a strong east–west geographical partition of mtDNA nucleotide sequences or haplotypes (Roehrdanz et al. 2003). The mtDNA boundary previously observed for northern corn rootworm populations runs in a roughly north-to-south direction, with the line of demarcation located in east central Illinois. The main boundary zone is narrow, and the eastern populations exhibit less overall variability than the western populations. Nine haplotypes were found east of the boundary; two of these halotypes accounted for 96% of the insects, but the other seven were recovered only once each. West of the boundary, 46 haplotypes were found. The most numerous haplotype accounted for 49% of the insects sampled and was concentrated in central Illinois (69% frequency). Phylogenetic trees based on genetic distance measurements of the mtDNA produced two distinct clades, A and B, with a genetic difference of ≈2.5%. Insects east and west of the boundary belonged to two distinct mtDNA clades. The data indicate that there has been little genetic mixing of mtDNA across this boundary (Roehrdanz et al. 2003).

Wolbachia species are intracellular bacteria with widespread distribution among insects, crustaceans, and nematodes (Werren 1997, Stouthamer et al. 1999). Depending on the detection methods used, *Wolbachia* species have been found in 20–76% of insects (Werren et al. 1995a, Jeyaprakash and Hoy 2000). *Wolbachia* infection is often associated with reproductive disruptions, including cytoplasmic incompatibility (CI), par-

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thenogenesis induction, feminization of males, and killing of male embryos. *Wolbachia* infection is maintained in a population via vertical transfer from mother to offspring. CI typically results in a level of embryonic mortality when *Wolbachia*-infected males mate with uninfected females. The reciprocal cross is compatible. This inheritance pattern can give the *Wolbachia*-infected females an advantage that allows *Wolbachia* infections to spread (McGraw and O'Neill 1999). The latter three *Wolbachia*-induced reproductive anomalies result in various degrees of female-biased sex ratios. Because both *Wolbachia* infection and mtDNA are maternally inherited, an expanding *Wolbachia* infection is typically accompanied by the mtDNA haplotype(s) of the females that were initially infected. This process, known as a *Wolbachia* "sweep", can lead to a significant reduction of mtDNA diversity in the infected populations (Turelli et al. 1992, Shoemaker et al. 1999, Behura et al. 2001, Armbruster et al. 2003). *Wolbachia* strain identification and phylogenetics have relied primarily on the comparisons of partial nucleotide sequences of two *Wolbachia* genes, *wsp* and *fts*Z (Werren et al. 1995b, Zhou et al. 1998).

In preliminary investigations, we detected *Wolbachia* in some populations of northern corn rootworm from east of the Mississippi River. A screen for *Wolbachia* infection in *Diabrotica* species established its widespread occurrence in the western corn rootworm, *Diabrotica virgifera virgifera* LeConte and found it in other species [*Diabrotica cristata* (Harris)] but not in northern corn rootworm (Clark et al. 2001). Because *Wolbachia* infections can establish breeding barriers, we investigated whether they could provide an explanation for the sharp mtDNA divide observed in the northern corn rootworm populations. *Wolbachia* infection of *D. virgifera virgifera* has been implicated maintaining the reproductive isolation of two subspecies, western corn rootworm and the Mexican corn rootworm, *Diabrotica virgifera zeae* Krysan & Smith (Giordano et al. 1997). In this study, we examine whether the reproductive isolation in northern corn rootworm as manifested by mtDNA haplotype discontinuity is linked to infection by *Wolbachia* bacteria. Knowledge of mechanisms that can affect genetic diversity of northern corn rootworm is of interest because of this species' status as an economically important pest of corn.

Materials and Methods

Rootworms. Adult northern corn rootworms were collected and handled as described in Roehrdanz et al. 2003. Many of the insects from that study also were used here along with newly collected insects from central Illinois. All of the collection sites are numbered and listed as part of Table 1. For each collection site, the number of insects that were used in the previous study (Roehrdanz et al. 2003) are listed in a separate column. The locations of the numbered sites are shown in Fig. 1. Insect and DNA samples are preserved at the USDA Biosciences Research Laboratory in Fargo, ND.

DNA Preparation and Polymerase Chain Reaction (PCR). Total DNA from adult rootworms was prepared as in the previous work (Roehrdanz et al. 2003). To determine the mitochondrial DNA clades of the insects, long PCR reactions of mitochondrial DNA segments, 12S-N4 (≈5.5 kb) and CB2H-C2R (≈7.2 kb) were done as described by Roehrdanz and Degrugillier (1998).

The presence of *Wolbachia* bacteria was determined by conducting PCR with primers specific for *Wolbachia* genes. Primers WOL-1 (5′-TTG TAG CCT GCT ATG GTA TAA CT) and WOL-2 (5′-GAA TAG GTA TGA TTT TCA TG) amplify a *Wolbachia*-specific portion of the 16S ribosomal RNA gene (O'Neill et al. 1992), and they were used as an initial test for the presence of *Wolbachia* bacteria. The primers *fts*Zf1 (5′-GTT GTC GCA AAT ACC GAT GC) and *fts*Zf2 (5′-CTT AAG TAA GCT GGT ATA TC) amplify ≈1,050 bp of the *fts*Z cell cycle protein (Werren et al. 1995b). *Fts*Z-F (5′-TAC TGA CTG TTG GAG TTG TAA CTA AGC CGT) and *fts*Z-R (5′-TGC CAG TTG CAA GAA CAG AAA CTC TAA CTC) amplify ≈570 bp in the middle of the larger *fts*Z segment (Jeyaprakash and Hoy 2000). An ≈600-bp piece of *wsp*, a *Wolbachia* surface protein gene, was amplified with primers *wsp*-81 F (5′-TGG TCC AAT AAG TGA TGA AGA AAC TAG CTA) and *wsp*-691R (5′-AAA AAT TAA ACG CTA CTC CAG CTT CTG CAC) (Zhou et al. 1998). The segment of the *wsp* gene includes the four hypervariable regions described by Baldo et al. (2005). The PCR conditions were 35 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 3 min. Once the 16S primers determined the presence of *Wolbachia* in several northern corn rootworm individuals, the *fts*Z and *wsp* gene segments were used to define the nature and extent of infection.

DNA Analysis. The rootworm mtDNA long PCR segments were cleaved with restriction enzymes and assigned to haplotypes as described previously (Roehrdanz et al. 2003). The *fts*Z long amplicon and the *wsp* gene segment were cloned from individuals obtained from opposite sides of the mtDNA boundary.

Six *fts*Z clones from northern corn rootworm and one from western corn rootworm were sequenced along with four northern corn rootworm and two western corn rootworm *wsp* clones. DNA sequencing was done by the Iowa State University DNA sequencing facility. GenBank accession numbers of *Wolbachia* sequences from this work are AF532961–63 and AY136551–554 for *fts*Z and AY138259–64 for *wsp*. DNA sequences were aligned using the AlignX component of VectorAdvance software package (Invitrogen, Carlsbad, CA).

Wolbachia DNA sequences from other insects that were most similar to the *Wolbachia* sequences reported here were obtained from GenBank via a BLAST search. Other GenBank sequences were obtained from the literature, and they were chosen to reflect a range of *Wolbachia* supergroup A and B strains. Because the GenBank sequences vary with respect to their endpoints, we trimmed the ends of our sequences (and sometimes the database sequences as well) to obtain a collection of coincident nucleotides.

Table 1. Northern corn rootworm collection sites and *Wolbachia* infection status

Site[a]	State	Location	MT clade[b]	Wol−	Wol+	Type 1	Type 2	ND[c]	*N* prev[d]	*N*[e]
1	ND	Fargo	A/B	5	0				5	5
2	SD	Brookings	A/B	10	0				9	10
3	KS	Chapman	A	5	0				5	5
4	IA	Ames	A	6	0				6	6
5	IA	Preston	A	10	0					10
6	WI	Arlington	A	9	0				8	9
7	WI	Janesville	A/B	9	1	1			8	10
8	IL	Manlius	A	6	0					6
9	IL	Annawan	A	1	0				1	1
10	IL	Hanover	A	5	1			1	6	6
11	IL	Palmyra	A	6	0					6
12	IL	Chana	A	6	0					6
13	IL	Polo	A	9	0					9
14	IL	Warren Co.	A	9	0				9	9
15	IL	Hebron	A	1	0					1
16	IL	La Fayette	A	6	0					6
17	IL	West Jersey	A	6						6
18	IL	Castleton	A	8	7		7			15
19	IL	Princeville	A	13	2		2			15
20	IL	Stark Co.	A	7	1		1		8	8
21	IL	Laura	A	14	2		2		1	16
22	IL	Buda	A	14	1		1			15
23	IL	Dover	A	2	10		10		12	12
24	IL	Ashton	A	3	13		13			16
25	IL	Malta	A	1	3		3			4
26	IL	Pingree Grove	A	0	3		2	1	2	3
27	IL	Sugar Grove	A	1	9		7	2	6	10
28	IL	Yorkville	A	0	5		5		3	5
29	IL	Kendall Co.	A	4	1			1	3	5
30	IL	St. Augustine	A/B	12	0				12	12
31	IL	Lasalle Co.	A	1	8		7	1	8	9
32	IL	La Prairie Center	A	2	13		11	2		15
33	IL	Varna	A	1	17		16	1	11	18
34	IL	Toluca	A	0	6		6			6
35	IL	Mazon	A	0	3		2	1		3
36	IL	Gardner	A	4	8		8		10	12
37	IL	Minonk	A	0	12		12		9	12
38	IL	Flanagan	A	1	23		20	3	17	24
39	IL	Panola	A	0	2		2		2	2
40	IL	Gridley	A	1	4		4		5	5
41	IL	Clarksville	A	4	29		13	16	22	33
42	IL	Kickapoo	A	14	1		1			15
43	IL	Brownwood	A	0	11		11			11
44	IL	Tazewell	A	1	5		5			6
45	IL	Midland City	A	3	9		9		12	12
46	IL	Hallsville	A	0	2		1	1	2	2
47	IL	Lincoln	A	1	20		8	12	16	21
48	IL	Saunemin	A	4	14		11	3	17	18
49	IL	Wing	A/B	1	16	12	1	3	9	17
50	IL	Cooksville	A/B	2	17	2	10	5	14	19
51	IL	Clinton	A/B	0	4	1	2	1	2	4
52	IL	Harristown	A/B	0	5	4	1		5	5
53	IL	Weldon	B	0	1	1			1	1
54	IL	Urbana	B	0	5	5			5	5
55	IL	Perdueville	B	0	11	6		5	11	11
56	IL	Bement	B	1	4	4			5	5
57	IL	Cerro Gordo	B	0	5	5			5	5
58	IL	Pierson/Atwood	B	0	7	5		2	7	7
59	IL	Arcola	B	2	3	3			5	5
60	IL	Armstrong	B	0	7	5		2	7	7
61	IN	Jasper Co.	B	0	2			2	2	2
62	IN	Newton Co.	B	0	2	2			2	2
63	IN	Tippecanoe Co.	B	0	2	2			2	2
64	IN	Wells Co.	B	0	2	2			2	2
65	OH	Miami Co.	B	0	2	2			2	2
66	PA	Rockspring	B	0	5	3		2	5	5
				231	346	65	214	67	326	577

[a] Site numbers correspond to map in Fig. 1.
[b] mtDNA clades found at site (see Roehrdanz et al. 2003 for description).
[c] *Wolbachia* type not determined.
[d] Number of insects that were part of the previous study (Roehrdanz et al. 2003).
[e] Total number of insects tested for *Wolbachia.*

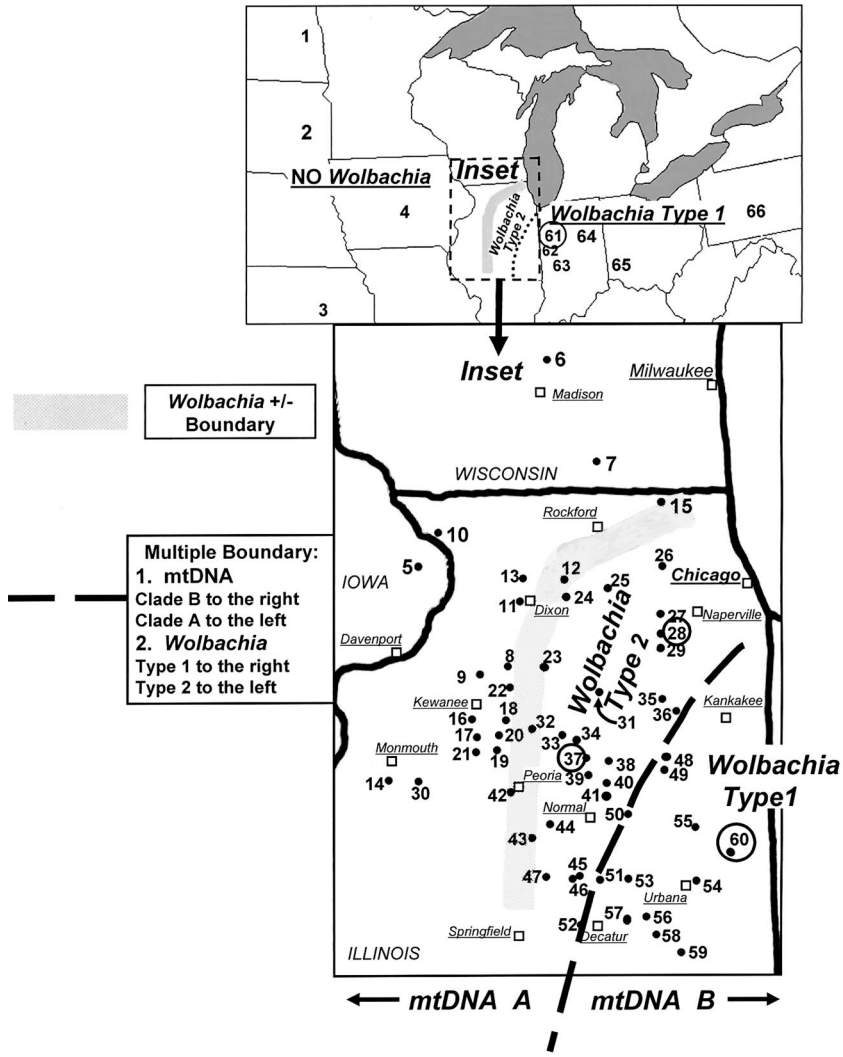

Fig. 1. Map of collection sites and *Wolbachia* strain boundaries in the central United States. The site numbers and associated information are listed in Table 1. Circled site numbers are the locations from which *Wolbachia* sequences were recovered (28 Yorkville, IL; 37 Minonk, IL; 60 Armstrong, IL; 61 Jasper County, IN) Labeled squares and underlined italics are the locations of cities included for orientation. State names are given in uppercase italics. The key boundaries in northern corn rootworm populations are drawn. The coincident mtDNA clade B versus clade A and the NCR *Wolbachia* Type 1 versus Type 2 boundary are indicated by a broken line. The less well defined *Wolbachia*-infected versus *Wolbachia*-uninfected is shown as a broad stippled line.

We constructed trees with 867 bp of *fts*Z and ≈550 bp of *wsp.* Tree construction was based on a sequence distance method and uses the neighbor joining (NJ) algorithm of Saitou and Nei (1987).

For the survey of *Wolbachia* distribution the presence of *Wolbachia* bacteria was determined by PCR amplification of either the *fts*Z short fragment or the *wsp* fragment. *Alu*I restriction fragment differences (634 and 228 bp for Type 1 versus 511 and 353 bp for Type 2) between the two types of cloned *Wolbachia* *fts*Z sequences were used to classify the *Wolbachia* from these individuals (data not shown).

The mtDNA haplotype of individuals used in the previous study is known. Newly collected beetles from central and western Illinois came from areas that were previously determined to contain >99% mtDNA clade A individuals (Roehrdanz et al. 2003). Rather than determine the detailed haplotypes for these insects, some restriction fragment length polymorphisms were used that had shown to be diagnostic for mtDNA clade A versus mtDNA clade B (data not shown).

Results

Wolbachia bacteria was detected in northern corn rootworm populations from the eastern portion of their range on both sides of the mtDNA genetic boundary in east central Illinois by using primers spe-

Table 2. Polymorphic positions in *Wolbachia ftsZ* sequences from northern and western corn rootworms

Wolbachia source[a]	Sequence position																																		
	27	28	33	39	42	45	99	138	144	353	361	376	544	545	565	582	633	639	642	654	663	774	865	868	874	879	905	906	907	912	933	954	965	967	994	1033	1035
wcr07552	X	X	X	X	X	X	X	C	A	T	C	C	T	G	G	C	C	A	G	T	G	A	G	A	G	C	G	T	C	T	G	A	T	C	G	X	X
wcr11271	X	X	X	X	X	X	X	C	A	T	C	C	G	A	G	C	C	A	G	T	G	A	G	A	G	C	G	T	C	T	G	A	T	C	G	X	X
wcrGR	G	T	G	A	A	C	C	C	A	T	C	C	G	A	G	C	C	A	G	T	G	A	A	A	G	C	G	T	C	T	G	A	T	C	G	A	C
ncr1ARa	G	T	G	A	A	C	C	C	A	T	C	C	G	A	G	C	C	A	G	T	G	G	G	A	G	C	G	T	C	T	G	A	T	T	G	A	C
ncr1ARb	G	T	G	A	A	C	C	C	A	T	C	C	G	A	G	C	C	A	G	T	G	A	G	A	G	C	G	T	C	T	G	A	T	C	G	A	C
ncr1IN	G	T	G	A	A	C	C	C	A	T	C	C	G	A	G	C	C	A	G	T	G	A	G	A	G	C	G	T	C	T	G	A	T	C	G	A	C
ncr2MN	A	C	A	T	G	T	T	T	G	C	T	T	G	A	A	T	T	G	A	C	A	A	G	G	A	T	-	-	-	C	A	C	C	C	A	G	T
ncr2YKa	A	C	A	T	G	T	T	T	G	C	T	T	G	A	A	T	T	G	A	C	A	A	G	G	A	T	-	-	-	C	A	C	C	C	A	G	T
ncr2YKb	A	C	A	T	G	T	T	T	G	C	T	T	G	A	A	T	T	G	A	C	A	A	G	G	A	T	-	-	-	C	A	C	C	C	A	G	T

Shaded boxes, majority nucleotide; X, sequence unavailable.

[a] *Diabrotica virgifera:* wcr07552 (AY007552) (Clark et al. 2001), wcr11271 (AF011271) (Giordano et al. 1997), wcrGR (AY136551) (Gresham, NE). *D. barberi:* ncr1ARa (AY136552) and ncr1ARb (AY136553) (Armstrong, IL); ncr1IN (AY136554) (Jasper County, IN); ncr2MN (AF532961) (Minonk, IL); ncr2YKa (AF532962); and ncr2YKb (AF532963) (Yorkville, IL).

cific for *Wolbachia* 16S rDNA, *fts*Z and *wsp* genes (Table 1; Fig. 1). Portions of the *Wolbachia ftsZ* and *wsp* genes were sequenced from two collection sites on each side of the mtDNA boundary. The 1,058-bp *ftsZ* sequences from northern corn rootworm can be segregated into two groups, designated NCR Type 1 and NCR Type 2. NCR Type 1 sequences were obtained from locations 60 (Armstrong, IL) and 61 (Jasper County, IN). Northern corn rootworm Type 2-carrying insects were obtained from locations 28 and 37 (Yorkville and Minonk, IL) (Fig. 1). Two *fts*Z clones were sequenced from the Yorkville and Armstrong insects and one clone each from Jasper and Minonk. A *Wolbachia fts*Z clone from a Nebraska-collected western corn rootworm also was sequenced. The polymorphic nucleotide positions in the *fts*Z clones are shown in Table 2. Included in the table are two western corn rootworm *fts*Z GenBank sequences from the work of Giordano et al. (1997) and Clark et al. (2001). Those two sequences are slightly shorter than the sequences we obtained. The *fts*Z sequences of NCR Types 1 and 2 differ by ≈3.1%, including one 3-bp deletion at nucleotides 905–907. The consensus NCR Type 1 *Wolbachia fts*Z sequence is identical to the consensus *fts*Z found in the congeneric western corn rootworm. The three NCR Type 2 sequences are identical.

For *wsp*, we sequenced single clones from the same four northern corn rootworm and one western corn rootworm individuals. An additional clone from a Brookings, SD, western corn rootworm individual also was sequenced. The ≈600-bp *wsp* sequences are also different in the two strains. As with the *fts*Z sequence, the 611-bp NCR Type 1 *wsp* sequence is essentially identical to that from western corn rootworm. One clone from northern corn rootworm and one from western corn rootworm have different single base substitutions. NCR Type 2 sequences are only 599 bp and differ from the NCR Type 1 by 63 substitutions and 15 indels (insertions or deletions of nucleotides), resulting in a combined sequence difference of ≈13%. The two NCR Type 2 clones are identical.

Table 3 has a comparison of the two most highly polymorphic sections of *wsp*, including the indels. The NCR Type 1 sequence codes for 200 amino acids compared with 196 for NCR Type 2. Including the deletions, there are 35 amino acid differences (0.823 similarity) between the *wsp* products of these two

Table 3. Alignment of the two most divergent regions of *wsp* from northern and western corn rootworm

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        209                                                   262
         |                                                     |
ncr2MNw TGGTTGAATAAAGATGC------AGATGTAGCAGGTGACACAG---------TT
ncr2YKw ................------..................---------..
ncr1ARw .ACC.A..C...A...ATGTTAA.......A..TT....C...CAAATACTA..
 wcrBRw .ACC.A..C...A...ATGTTAA.......A..TT....C...CAAATACTA..
 wcrGRw .ACC.A..C...A...ATGTTAA.......A..TT....C...CAAATACTA..
ncr1JSw .ACC.A..C...A...ATGTTAA.......A..TT....C...CAAATACTA..
        507                                                   560
         |                                                     |
ncr2MNw ACGGTGCTAATTTCGATAAAACTTCTGGTGCGACCGGTAAAGATAAAGGAGGACATACA
ncr2YKw ............................................................
ncr1ARw TT......C....T......GA.G..T---.C..A..C.....C.....G.A..TC.A.
 wcrBRw TT......C....T......GA.G..T---.T..A..C.....C.....G.A..TC.A.
 wcrGRw TT......C....T......GA.G..T---.T..A..C.....C.....G.A..TC.A.
ncr1JSw TT......C....T......GA.G..T---.T..A..C.....C.....G.A..TC.A.
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Nucleotide position numbers above the sequences. Dot indicates identical nucleotide in all sequences relative to ncr2MN. Dash indicates deletion relative to other sequences. ncr2MNw (AY138263) (Minonk, IL), ncr2YKw (AY138264) (Yorkville, IL), ncr1ARw (AY138259) (Armstrong, IL), ncr1JSw (AY138262) (Jasper County, IN), wcrGRw (AY138261) (Gresham, NE), and wcrBRw (AY138260) (Brookings, SD).

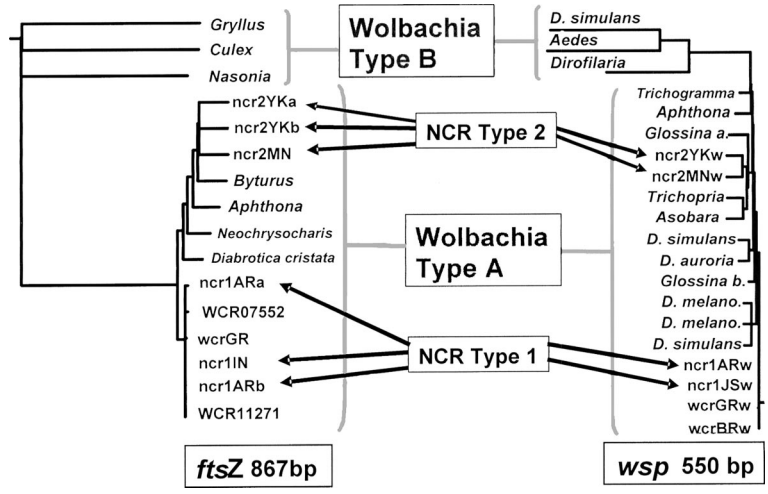

Fig. 2. Phylogenetic comparison of northern corn rootworm *Wolbachia ftsZ* and *wsp* sequences with members of *Wolbachia* supergroup A and supergroup B. *Wolbachia* hosts: ftsZ – *Diabrotica barberi* - ncr2YKa (AF532962) and ncr2YKb (AF532963), Yorkville, IL, map location 28; ncr2MN (AF532961), Minonk, IL, map location 37; ncr1ARa (AY136552) and ncr1ARb (AY136553), Armstrong, IL, map location 60; ncr1IN (AY136554), Jasper County, IN, map location 61. *D. virgifera* - wcrGR (AY136551), Gresham, NE; WCR11271 (AF011271, Giordano et al. 1997); WCR07552 (AY007552, Clark et al. 2001). Other species from the GenBank database – AF011269 (*Gryllus integer*); U28209 (*Culex pipiens*); U28205 (*Nasonia vitripennis*); AJ250964 (*Byturus tomentosus*); AY136550 (*Aphthona nigriscutis*); AB037896 (*Neochrysocharis formosa*); AY007556 (*Diabrotica cristata*); wsp - *Diabrotica barberi* - ncr2YKw (AY138264), Yorkville, IL; ncr2MNw (AY138263)), Minonk, IL; ncr1ARw (AY138259), Armstrong, IL; ncr1JSw (AY138262) Jasper County, IN. *D. virgifera* - wcrGRw (AY138261), Gresham, NE; wcrBRw (AY138260), Brookings, SD. Other species from the GenBank database – AF539860 (*Aphthona nigriscutis*; AJ580923, AF020070, AF020067 (*Drosophila simulans*); AF338346, AF020065 (*D. melanogaster*); AF020062 (*D. auroria*); AF071910 (*Trichopria drosophilae*); AF124856 (*Asobara tabida*), AF020077 (*Glossina austeni*); AF165685 (*G. brevipalpus*); AJ580921 (*Dirofilaria immitis*); AF452645 (*Trichogramma brassicae*); AJ580922 (*Aedes albopictus*).

Wolbachia strains for the interval examined. Baldo et al. (2005) have characterized this portion of the *Wolbachia wsp* gene as being composed of four hypervariable regions interspersed with more conserved segments. The two regions shown in Table 3 correspond to what they have designated as hypervariable region two and hypervariable region 4.

Most of the *Wolbachia* strains derived from insects can be divided into two major supergroups, A and B (Lo et al. 2002). To determine to which supergroup our sequences belonged, we conducted a distance-based phylogenetic analysis by using 867 bp of the *fts*Z and ≈550 bp of the *wsp* genes. We compared our sequences with sequences from the GenBank database from each of the two supergroups. The *fts*Z and *wsp* sequences from both the NCR Types 1 and 2 cluster within the *Wolbachia* supergroup A (Fig. 2).

We measured the distribution of *Wolbachia* in northern corn rootworm by using two different parameters, geographical location and mtDNA haplotypes. In total, 326 northern corn rootworm adults with known mtDNA haplotypes were tested for *Wolbachia* infection, among them 89 from east of the mtDNA boundary and 237 from west of the boundary. These insects were all part of the previous mtDNA diversity study (Roehrdanz et al. 2003). New collections were acquired from west of the mtDNA boundary in Illinois. From these collections, 251 individuals were tested for *Wolbachia* infection. The first 85 of these individuals tested by restriction fragment length polymorphism, all belonged to mtDNA clade A. The remaining individuals from the same localities were not specifically tested for mtDNA clade, but nearly all of them are likely to be members of clade A based on the combined observations of this and the previous study (Roehrdanz et al. 2003) that 325 of 329 (98.8%) northern corn rootworm collected west of the mtDNA boundary in Illinois in this region. have belonged to clade A. Differentiation between *Wolbachia* strains NCR Type 1 and Type 2 was based on the restriction fragment length polymorphism patterns of *fts*Z amplicon by using the *Alu*I restriction enzyme. For some individuals, especially along the mtDNA boundary, the restriction fragment length polymorphism pattern was ambiguous, and their *Wolbachia* type could not be assigned.

The incidence of *Wolbachia* infection by collection site is presented in Table 1. The relative locations of the 66 collection sites are shown in Fig. 1. *Wolbachia* infection rate declines from east to west in Illinois. Starting in the middle of Fig. 1 at sites 33 and 34, 23/23 (100%) tested positive for *Wolbachia* bacteria. At site 32, 13/15 (87%) have *Wolbachia* bacteria, whereas site 46 has 7/15 (47%). Sites 41, 42, 47, 48, and 49 combined have *Wolbachia* bacteria in 6/48 (12.5%). Finally, farthest west in Illinois, sites 22 and 53 had no *Wolbachia* bacteria (0/21). No *Wolbachia* infection has been detected in northern corn rootworms collected west of the Mississippi River, which forms the boundary between Illinois and Iowa. This includes both the

Table 4. Mitochondrial haplotypes and *Wolbachia* infection status in the northern corn rootworm

mtDNA haplotype[a]	Clade[a]	Infection status		NCR *Wolbachia*			Total
		Wol−	Wol+	Type 1	Type 2	ND[b]	
H1	A	6					6
H2	A	38	125		105	20	163
H8	A	11					11
H6	A	4					4
H14	A	8	1			1	9
H15	A		12		4	8	12
H23	A	4					4
H29	A	9	8		6	2	17
Other "A" combined[c]	A	15	16		14	2	31
"A" undefined[d]	A	4	81		65	16	85
H30	B	6					6
H38	B	4	44	34		10	48
H44	B	4	33	27		6	37
Other "B" combined[c]	B		4	4			4
Subtotal mt clade A		99	243		194	49	342
Subtotal mt clade B		14	81	65	0	16	95
Undetermed mt clade[e]		118	22		20	2	140
Total		231	346	65	214	67	577

[a] mtDNA haplotypes and clades defined in Roehrdanz et al. (2003).
[b] *Wolbachia* strain type not determined.
[c] Haplotypes made up of less than four individuals were combined.
[d] Complete mtDNA haplotypes were not determined, but diagnostic RFLPs confirmed that they were all from clade A.
[e] mtDNA not examined. All individuals were collected in Illinois from west of the mtDNA boundary.

results described here and those of Clark et al. 2001. The broad stippled band in Fig. 1 indicates the approximate location of a *Wolbachia* ± boundary in central Illinois. That boundary is ≈120 km west of the mtDNA dividing line, and it is best described as a declining gradient of infection. The decline in *Wolbachia* infections across this region parallels a decline in the frequency of mtDNA haplotype 2.

The distribution of *Wolbachia* strains among the mtDNA haplotypes is presented in Table 4. Approximately 85% of the mtDNA clade B individuals were infected with *Wolbachia* bacteria, primarily in haplotypes 38 and 44. No clearly identifiable NCR Type 2 *Wolbachia* bacteria were observed in this group. Among the beetles that were confirmed to be mtDNA clade A individuals, 70.7% were infected with *Wolbachia* bacteria. Most of these individuals belonged to haplotypes 2, 15, and 29. If the undetermined but presumptive clade A insects are included the infection rate drops to 56%. All of the 214 identified *Wolbachia* isolates from clade A insects were strain NCR Type 2.

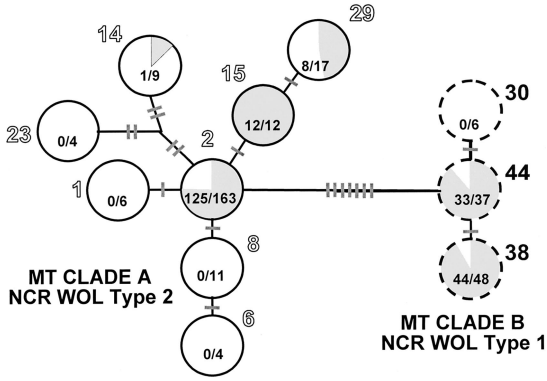

Fig. 3. Partial mtDNA haplotype network showing frequency of *Wolbachia* infection among the haplotypes listed in Table 4. Large numbers outside the circles are mitochondrial haplotypes. Bars on connecting lines indicate the minimal number of restriction site changes (Roehrdanz et al. 2003). Fractions inside circle are number of *Wolbachia*-infected versus total number tested. That fraction is also represented by the shaded area of the circles.

Figure 3 contains a haplotype network of the more common haplotypes (four or more individuals) identified here. It is derived from a similar network in our previous work (Roehrdanz et al. 2003. Haplotype 2 is the dominant haplotype in the clade A portion of the network. Among the fully identified mtDNA clade A haplotypes tested for *Wolbachia* bacteria, 71% are haplotype 2. The branch including haplotypes 2, 15, and 29 has an infection rate of 75.5%. By contrast, the other branches of clade A contained only one infected individual out of 27 (3.7%). In clade B, insects with haplotypes 38 and 44 have a high infection rate (90.1%), but individuals with haplotype 30 seem to be uninfected. *Wolbachia* sequences indicate that each type resides mostly in only one of the previously reported mtDNA clades. Thus, the two identified *Wolbachia* strains seem to segregate with the respective mtDNA clades that define the mtDNA genetic boundary in eastern Illinois (Fig. 3).

Discussion

We found *Wolbachia* infection to be common in northern corn rootworm collected from Illinois to Pennsylvania. The screen for *Wolbachia* by Clark et al. (2001) in several species of *Diabrotica* failed to find any evidence of infection in northern corn rootworm. This is likely a consequence of their restricted sam-

pling to areas west of the Mississippi River. All of the *Wolbachia*-infected individuals in this work were found east of the Mississippi. In agreement with Clark et al. (2001), we did not find *Wolbachia* in any of the insects from five sample sites west of the Mississippi River. Even a couple sites just east of the Mississippi (14 and 30; Table 1; Fig. 1) produced no infected insects. In our previous work (Roehrdanz et al. 2003), we found a small cluster of clade B mtDNA haplotypes in the eastern Dakotas that was dominated by haplotype 30. In keeping with the geographic limits of *Wolbachia* distribution, the haplotype 30 individuals we tested do not have *Wolbachia* infection. This compares with the 90% infection rate of clade B individuals from east of the Illinois mtDNA boundary. We are reminded of the need to sample across the geographic range of any species to determine the degree of *Wolbachia* infection. Conversely, the *Wolbachia* status of any species cannot be extrapolated beyond the populations actually tested.

The presence of at least two divergent *Wolbachia* strains on opposite sides of a sharply defined mtDNA haplotype boundary in eastern Illinois suggests that the two *Wolbachia* strains are incompatible, and little, if any, mtDNA introgression occurs between these two infected populations. The distribution of the *Wolbachia* strains has the effect of delineating a third identifiable population of northern corn rootworm. To the east are northern corn rootworm clade B mtDNA beetles, primarily haplotypes 38 and 44, infected with NCR Type 1 *Wolbachia.* In central Illinois are northern corn rootworm clade A mtDNA beetles, primarily haplotype 2, infected with NCR Type 2 *Wolbachia.* Finally, west of central Illinois are northern corn rootworm clade A beetles, and a few northern corn rootworm clade B beetles with diverse haplotypes and with no *Wolbachia* (Fig. 1). There are no apparent morphological differences between the two clades. In all likelihood the geographical position of the mtDNA–*Wolbachia* Type 1 and 2 boundary is an accident of history. Two separate *Wolbachia* infections occurred in northern corn rootworm with each spreading at its own rate until the mutually incompatible infections collided and the boundary was established. Because the northern corn rootworm collections were obtained in a narrow window of only a few years, we currently have no evidence that this boundary is being pushed in either direction. A follow-up survey is planned for the future to determine whether the boundary positions are dynamic. Two general schemes might be considered to account for any future movement of this boundary. Such movement could be the result of unpredictable changes in the local environment that favors one of the two populations. Alternatively, the reproductive incompatibility of the two *Wolbachia* strains may not be equal, giving one of the two stains a small but persistent advantage in interstrain matings, resulting in a constant pressure in one direction.

The boundary between the NCR Type 2 *Wolbachia*-infected beetles and uninfected beetles seems to be less distinct, suggesting that this boundary may still be changing. Because *Wolbachia* is well known for its ability to spread into uninfected populations, we would predict that the infection would expand westward into uninfected northern corn rootworm populations. However, not all *Wolbachia* infections spread rapidly. The congeneric corn rootworm subspecies western corn rootworm and Mexican corn rootworm are divided by *Wolbachia*-based CI (Giordano et al. 1997). Western corn rootworm harbors *Wolbachia* bacteria, but Mexican corn rootworm does not. Regardless, the apparent subspecies boundary has not moved markedly at least during the latter half of the 20th century (Krysan and Smith 1987). The current northern corn rootworm *Wolbachia* boundary locations are based on 1998–2001 insect collections. There are no obvious geological features associated with either boundary. Adult insects can fly and infected beetles should be capable of some movement to the west despite the prevailing winds flowing from west to east. Repeated collections over a longer time frame than we have used would be necessary to detect any measurable movement of the northern corn rootworm boundaries.

The two *Wolbachia* types observed both belong to supergroup A, but they are sufficiently divergent to be considered separate strains. A BLAST search of GenBank by using the NCR Type 2 *fts*Z nucleotide sequence returned a sequence from the beetle *Byturus tomentosus* (De Geer) (Malloch et al. 2000) as nearly identical (0.1% difference) over 957 bp, but this is only part of one gene from the entire *Wolbachia* genome. Other genes from both northern corn rootworm and *Byturus Wolbachia*-infected individuals would need to be examined to more firmly establish a link between those two strains.

Although maternal inheritance perpetuates *Wolbachia* infections, the evidence indicates that *Wolbachia* bacteria enter a species through horizontal transfer from another species (Ono et al. 2001, Jiggins et al. 2002, Mitsuhashi et al. 2002, Tsutsui et al. 2003). We were surprised that the northern corn rootworm *Wolbachia* found from eastern Illinois to Pennsylvania seems to be the same strain of *Wolbachia* as the *Wolbachia* from western corn rootworm for both *fts*Z and *wsp.* Could the eastern population of northern corn rootworm have acquired this *Wolbachia* strain via transfer from its close congener, western corn rootworm? The two species are currently very similar in life cycle and feeding preferences, and they display extensive sympatry. Therefore, opportunities for close interactions abound and the potential for transfer seems plausible. There are no data to support vertical transfer via interspecific hybridization. The genetic divergence between western corn rootworm and northern corn rootworm mtDNA is twice the divergence between the two major northern corn rootworm clades (Szalanski et al. 2000, Roehrdanz et al. 2003); thus, the Type 1 *Wolbachia*-infected northern corn rootworms do not contain western corn rootworm-like mtDNA. The argument against horizontal transfer resides in the historical records of the distribution of *D. barberi* and *D. virgifera.* Krysan and Smith

(1987) place the range of northern corn rootworm as covering approximately the northeastern quadrant of the United States. The current range of western corn rootworm includes much of that same area and extends south and west to southern Mexico. However, the entire eastern expanse of western corn rootworm is the result of a recent invasion proceeding from west to east. Western corn rootworm was not reported from east of the Mississippi River until the second half of the 20th century, with the first report from Illinois coming in 1964 (Petty 1965). If northern corn rootworm were to have acquired *Wolbachia* Type 1 directly from western corn rootworm, for example, through a common parasitoid, the entire process of infection, geographic spread, and establishment of the boundary zone would have to have occurred in <35 yr. That seems like a very short time period for an infection that is spread via the reproductive cycle and not external contact in a univoltine species. The >2% mtDNA genetic divergence between clades A and B implies that their separation is very old, perhaps on the order of a million years (Brower 1994). In the absence of any evidence that the NCR Type 1 strain originated recently from western corn rootworm or vice versa, the similarity of the strains might be coincidental. After all, there is no demonstrable connection between northern corn rootworm and *B. tomentosus*, yet their *Wolbachia* sequences are very similar. The complete genome of an insect-borne *Wolbachia* strain has been sequenced (Wu et al. 2004), and the availability of many more genes may help define their intrastrain evolutionary pathways. Baldo et al. (2006) have recently proposed a more sophisticated system of categorizing *Wolbachia* strains that uses sequences from multiple loci.

One practical concern is whether such a dramatic delineation of population diversity influenced by different *Wolbachia* strains could be accompanied by heritable traits that might differentially affect control programs. Insecticide resistance, extended diapause, and other phenotypes all pose a threat to current control technologies. Even in the presence of mutually incompatible strains of *Wolbachia*, whether *Wolbachia* can act as a barrier to nuclear gene flow and possibly as a speciation agent is still subject to debate. Unidirectional CI combined with another isolation mechanism may push populations to diverge (Shoemaker et al. 1999, 2003). Telschow et al. (2002) have shown that when bidirectional CI is present, reduced gene flow, genetic divergence, and even speciation may be possible. Alternatively, Ballard et al. (2002) present evidence that *Wolbachia* generated mtDNA differentiation does not lead to reduced gene flow and nuclear gene differentiation in a fruit fly. In another dipteran, it is reported that *Wolbachia*-associated mtDNA sweeps do not correlate with nuclear genetic differentiation (Baudry et al. 2003). An examination of the level of introgression in populations of northern corn rootworm by using polymorphic nuclear genetic markers could be used to determine the impact of presumptively incompatible *Wolbachia* infections on nuclear gene flow.

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References Cited

Armbruster, P., W. E. Damsky, R. Giordano, J. Birungi, L. E. Munstermann, and J. E. Conn. 2003. Infection of new- and old-world *Aedes albopictus* (Diptera: Culicidae) by the intracellular parasite *Wolbachia:* implications for host mitochondrial DNA evolution. J. Med. Entomol. 40: 356–360.

Baldo, L., N. Lo, and J. H. Werren. 2005. Mosaic nature of the *Wolbachia* surface protein. J. Bacteriol. 187: 5406–5418.

Baldo, L., J. C. Hotopp, K. A. Jolley, S. R. Bordenstein, S. A. Biber, R. R. Choudhury, C. Hayashi, M. C. Maiden, H. Tettelin, and J. H. Werren. 2006. A multilocus sequence typing system for the endosymbiont *Wolbachia.* Appl. Environ. Microbiol. 72: 7098–7110.

Ballard, J.W.O., B. Chernoff, and A. C. James. 2002. Divergence of mitochondrial DNA is not corroborated by nuclear DNA, morphology, or behavior in *Drosophila simulans.* Evolution 56: 527–545.

Baudry, E., J. Bartos, K. Emerson, T. Whitworth, and J. Werren. 2003. *Wolbachia* and genetic variability in the birdnest blowfly *Protocalliphora sialia.* Mol. Ecol. 12: 1843–1854.

Behura, S. K., S. C. Sahu, M. Mohan, and S. Nair. 2001. *Wolbachia in the Asian rice gall midge, Orseolia oryzae* (Wood-Mason): correlation between host mitotypes and infection status. Insect Mol. Biol. 10: 163–171.

Brower, A.V.Z. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proc. Natl. Acad. Sci. U.S.A. 91: 6491–6495.

Clark, T. C., L. J. Meinke, S. R. Skoda, and J. E. Foster. 2001. Occurrence of *Wolbachia* in selected diabroticite (Coleoptera: Chrysomelidae) beetles. Ann. Entomol. Soc. Am. 94: 877–885.

Giordano, R., J. J. Jackson, and H. M. Robertson. 1997. The role of *Wolbachia* in reproductive incompatibilities and hybrid zones of *Diabrotica* beetles and *Gryllus* crickets. Proc. Nat. Acad. Sci. U.S.A. 94: 11439–11444.

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.

Jiggins, F. M., J. K. Bentley, M.E.N. Majerus, and G.D.D. Hurst. 2002. Recent changes in phenotype and patterns of specialization in *Wolbachia* bacteria. Mol. Ecol. 11: 1275–1283.

Krysan, J. L., and R. F. Smith. 1987. Systematics of the *virgifera* species group of *Diabrotica* (Coleoptera: Chrysomelidae: Galerucinae). Entomography 5: 375–484.

Lo, N., M. Casiraghi, E. Salati, C. Bazzocchi, and C. Bandi. 2002. How many *Wolbachia* supergroups exist? Mol. Biol. Evol. 19: 341–346.

Malloch, G., B. Fenton, and D. J. Butcher. 2000. Molecular evidence for multiple infections of a new subgroup of *Wolbachia* in the European raspberry beetle *Byturus tomentosus.* Mol. Ecol. 9: 77–90.

McGraw, E. A., and S. L. O'Neill. 1999. Evolution of *Wolbachia pipientis* transmission dynamics in insects. Trends Microbiol. 7: 297–302.

Mitsuhashi, W., T. Saiki, W. Wei, H. Kawakita, and M. Sato. 2002. Two novel strains of *Wolbachia* coexisting in both species of mulberry leafhoppers. Insect Mol. Biol. 11: 577–584.

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.

Ono, M., H. R. Braig, L. E. Munstermann, C. Ferro, and S. L. O'Neill. 2001. *Wolbachia* infections of phlebotomine sand flies (Diptera: Psychodidae). J. Med. Entomol. 38: 237–241.

Petty, H. B. 1965. Western corn rootworms were found for the first time this past year (1964) in Illinois just south of Rock Island. Insect situation, pp. 48–59. *In* Proceedings of the 17th Illinois Custom Spray Operators' Training School, 20–21 January 1965, University of Illinois, Champaign-Urbana, IL.

Roehrdanz, R. L., and M. E. Degrugillier. 1998. Long sections of mitochondrial DNA amplified from fourteen orders of insects using conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 91: 771–778.

Roehrdanz, R. L., A. L. Szalanski, and E. Levine. 2003. Mitochondrial DNA and ITS1 differentiation in geographical populations of northern corn rootworm, *Diabrotica barberi* (Coleoptera: Chrysomelidae): identification of distinct genetic populations. Ann. Entomol. Soc. Am. 96: 901–913.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing guide trees. Mol. Biol. Evol. 4: 406–425.

Shoemaker, D., V. Katju, and J. Jaenike. 1999. *Wolbachia* and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria.* Evolution 53: 1157–1164.

Shoemaker, D. D., G. Keller, and K. G. Ross. 2003. Effects of *Wolbachia* on mtDNA variation in two fire ant species. Mol. Ecol. 12: 1757–1771.

Stouthamer, R., J. A. Breeuwer, and G. D. Hurst. 1999. *Wolbachia pipientis:* microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53: 71–102.

Szalanski, A. L., R. L. Roehrdanz, and D. B. Taylor. 2000. Genetic relationship among five *Diabrotica* (Coleoptera: Chrysomelidae) based on rDNA and mtDNA sequences. Fla. Entomol. 83: 262–267.

Telschow, A., P. Hammerstein, and J. Werren. 2002. The effect of *Wolbachia* on genetic divergence between populations: models with two-way migration. Am. Nat. 160: S54–S66.

Tsutsui, N. D., S. N. Kauppinen, A. F. Oyafuso, and R. K. Grosberg. 2003. The distribution and evolutionary history of *Wolbachia* infection in native and introduced population of the invasive Argentine ant (*Linepithema humile*). Mol. Ecol. 12: 3057–3068.

Turelli, M., A. A. Hoffmann, and S. W. McKechnie. 1992. Dynamics of cytoplasmic incompatibility and mtDNA variation in natural *Drosohpila simulans* populations. Genetics 132: 713–723.

Werren, J. H. 1997. Biology of *Wolbachia.* Annu. Rev. Entomol. 42: 587–609.

Werren, J. H., D. Windsor, and L. Guo. 1995a. Distribution of *Wolbachia* among Neotropical arthropods. Proc. R. Soc. Lond. B 262: 197–204.

Werren, J. H., W. Zhang, and L. R. Guo. 1995b. Evolution and phylogeny of *Wolbachia:* reproductive parasites of arthropods. Proc. R. Soc. Lond. B 261: 55–71.

Wu, M., L. V. Sun, J. Vamathevan, M. Riegler, R. Deboy, J. C. Brownlie, E. A. McGraw, W. Martin, C. Esser, N. Ahmadinejad, et al. 2004. Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biol. 2: E69.

Zhou, W., F. Rousset, and S. L. O'Neill. 1998. Phylogeny and PCR-classification on of *Wolbachia* strain using *wsp* gene sequences. Proc. R. Soc. Lond. Ser. B 265: 509–551.

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