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Occurrence of *Wolbachia* in Selected Diabroticite (Coleoptera: Chrysomelidae) Beetles

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ABSTRACT *Wolbachia* are a rickettsial type bacteria that have been implicated as a cause of reproductive disruption and alteration in many insect species. Polymerase chain reaction assays of the 16S rRNA gene were conducted to reveal the prevalence of *Wolbachia* in 14 Diabroticite species, 12 *Diabrotica*, and two *Acalymma.* Assays revealed the presence of *Wolbachia* infection in three *Diabrotica* species; *D. virgifera virgifera* LeConte (all 40 individuals), *D. cristata* (Harris) (three of 25 individuals), *D. lemniscata* LeConte (all 20 individuals), and the two *Acalymma* species, *A. blandulum* (LeConte) (all 20 individuals), *A. vittatum* (F.) (one of 20 individuals). The identity of the *Wolbachia* strain was determined by sequencing the 16S rRNA and *fts*Z genes as well as a restriction enzyme digest of the *fts*Z gene. Distances and neighbor-joining trees on the Kimura 2-parameter measure as well as BlastN searches in GenBank revealed that the strain of *Wolbachia* infecting *D. lemniscata*, *D. v. virgifera*, *A. blandulum*, and *A. vittatum* are most likely the same strain of *Wolbachia* within the division A group. The strain of *Wolbachia* infecting *D. cristata* also clusters within the A group, but it is different from that found in the other four Diabroticites. Implications of these infections are also discussed.

KEY WORDS *Wolbachia*, *fts*Z, *Diabrotica*, *Acalymma*, rootworms, Diabroticites

Wolbachia IS A genus of rickettsial-type bacteria that are maternally inherited and infect numerous arthropod species. *Wolbachia* has been found to infect species within all of the major insect orders (Werren et al. 1995a, 1995b). These intercellular bacteria generally disrupt the reproductive biology of their hosts (Werren 1997). The reproductive effects caused by *Wolbachia* infection include induction of parthenogenesis in wasps (Stouthamer et al. 1993), feminization of male isopods (Bouchon et al. 1998), and cytoplasmic incompatibility (Yen and Barr 1973, Hoffmann and Turelli 1997, Hoffmann et al. 1998). The most studied of these effects is cytoplasmic incompatibility, which occurs between sperm and egg and generally takes two forms, unidirectional and bidirectional, that can result in zygotic death (Werren 1997). Unidirectional incompatibility occurs when infected sperm fertilizes an uninfected egg, however, the reciprocal cross is usually compatible. Bidirectional incompatibility occurs when male and female are infected with different stains of *Wolbachia*, which results in crosses in both directions being incompatible (Perrot-Minnot et al. 1996). Cytoplasmic incompatibility may result in a reproductive advantage for infected hosts resulting in a rapid spread of *Wolbachia* through insect populations (Turelli and Hoffmann 1991).

The galerucine chrysomelids known as Diabroticites in the tribe Luperini, subtribe Diabroticina, are a neotropical group of phytophagous beetles containing many species of economic importance (Smith and Lawrence 1967). Two genera within this group, *Acalymma* and *Diabrotica*, contain the most important pest species. For example, three *Diabrotica* species, *D. virgifera virgifera* LeConte, the western corn rootworm; *D. barberi* Smith & Lawrence, the northern corn rootworm; and *D. undecimpunctata howardi* Barber, the southern corn rootworm may have an annual $1 billion impact in terms of control costs and yield losses to United States maize producers (Metcalf 1986). An additional $100 million dollars may be lost to other *Diabrotica* such as *D. balteata* LeConte, *D. u. howardi*, and *D. u. undecimpunctata* Mannerheim due to attack on Cucurbitaceae and Fabaceae crops, such as cucumbers and peanuts (Metcalf et al. 1962, Metcalf 1986).

Degrugillier et al. (1991) provided the first anecdotal evidence that *Wolbachia* may indeed infect Diabroticites as they observed *Rickettsia*-like organisms in the testes and spermathecae of *D. v. virgifera.* Despite these observations, the organism was not identified. Giordano et al. (1997) identified these *Rickettsia*-like organisms as *Wolbachia* using polymerase chain reaction (PCR) amplification and subsequent sequencing of 16 rRNA and *fts*Z gene sequences. They also demonstrated the role of these bacteria in unidirectional reproductive isolation between infected *D. v. virgifera* and its uninfected subspecies *D. v. zeae*

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Table 1. List and number of Diabroticite species and specimens surveyed and distribution information

Species	General distribution	*n*	Code
D. barberi Smith & Lawrence	New Brunswick and Georgia to North Dakota and Oklahoma (Krysan and Smith 1987)	50	BAR
D. cristata (Harris)	USA east of Rocky Mountains, South to plateau of Mexico (Krysan and Smith 1987)	25	CRI
D. lemniscata LeConte	Arizona, New Mexico, Texas to Guatemala (Krysan and Smith 1987)	20	LEM
D. longicornis (Say)	Nebraska and Arizona to Durango and San Louis Potosi, Mexico (Krysan and Smith 1987)	25	LON
D. porracea Harold	Sonora, Mexico to Venezuela (Krysan and Smith 1987)	10	POR
D. virgifera virgifer LeConte	New York and Delaware to Idaho, western Texas, and Durango, Mexico (Krysan and Smith 1987)	40	VVI
D. vigifera zeae Krysan & Smith	Oklahoma to Central America (Krysan and Smith 1987)	20	VZE
D. viridula (F.)	Central Mexico to Bolivia, Peru, and Brazil (Krysan and Smith 1987)	20	VIR
D. balteata LeConte	southern United States from California to North Carolina to Columbia (Smith 1966, Krysan 1986)	20	BAL
D. speciosa Germar	South America (Krysan 1986)	16	SPE
D. undecimpunctata howardi Barber	southern Canada to northern Mexico primarily east of the Rocky Mountains (Smith 1966, Krysan 1986)	30	UHO
D. undecimpunctata undecimpunctata Mannerheim	British Columbia to Baja California primarily west of the Cascade and Sierra Nevada mountain ranges (Smith 1966, Krysan 1986)	15	UUN
A. Blandulum (LeConte)	Yucatan, Mexico to Nebraska and Arizona (Munroe and Smith 1980, Golden 1990)	20	BLA
A. vittatum (F.)	Manitoba and New Brunswick, Canada to Texas and Florida (Munroe and Smith 1980)	20	VIT

Krysan & Smith as curing individual beetles from *Wolbachia* infection led to the disappearance of reproductive incompatibility between these subspecies. Other than *Wolbachia* infection status, the only apparent difference between these subspecies is geographic range and color as their host status, life history and sex pheromone are nearly identical (Krysan et al. 1980, Krysan and Smith 1987). Similar reproductive incompatibilities have been observed between other closely related Diabroticites. For example, the morphological sibling species, *D. longicornis* (Say) and *D. barberi*, successfully hybridize ≈65% of the time when *D. barberi* males mate with *D. longicornis* females. However, reciprocal crosses produce offspring in only ≈5% of such crosses (Krysan et al. 1983; T.L.C., unpublished data). Despite these observations, the mechanism for this incompatibility remains unknown. There is no information on the presence of *Wolbachia* in other Diabroticites other than *D. v. virgifera.* The purpose of this study was to determine whether *Wolbachia* infections are implicated in other reproductive incompatibilities between closely related Diabroticites and to survey the extent of infection for selected species within this group.

Materials and Methods

Insects and DNA Extraction. Diabroticite beetles representing 12 *Diabrotica* and two *Acalymma* species (Table 1) were collected from several localities for evaluation (Table 2; Fig. 1). Specimens were identified using available dichotomous keys (Munroe and Smith 1980, Krysan 1986, Krysan and Smith 1987) with representative vouchers verified by J. L. Krysan (USDA-ARS retired) and preserved in 95% ethanol or frozen (−80°C). DNA was extracted using a modification of Black and DuTeau's (1997) CTAB (hexadecyltrimethylammonium bromide) extraction protocol. Individual beetles were ground in 500 μl CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 0.02 M EDTA (pH 8.0), 2.0% CTAB, and 0.2% β-mercaptoethanol), 5 μl of 20 μg/μl of proteinase K was then added to each sample. After vortexing the mixture, samples were held at 65°C for 1 h (vortexing at 20-min intervals). Samples were cooled to room temperature before adding 15 μl of 50 μg/ul RNase A, vortexing, and incubation at 37°C for 2.5 h (vortexing at 30-min intervals). After incubation with RNase A, samples were centrifuged at 10,000 × *g* for 5 min at room temperature. The supernatant was transferred to a fresh tube, where 500 μl of chloroform:isoamyl alcohol (24:1) was added. The mixture was vortexed and then centrifuged for 15 min at 10,000 × *g*. The upper aqueous layer was then transferred to a fresh tube where DNA was precipitated with 500 μl of 100% isopropanol (-20°C). The mixture was gently inverted 5 times and placed at 4°C for at least 2 h followed by centrifugation at 10,000 × *g* at 4°C for 30 min. The supernatant was removed and the DNA pellet was washed with 700 μl of 70% and 100% ethanol (-20°C), respectively. After the final wash and ethanol removal, the DNA pellet was air dried and resuspended overnight in 100 μl of TE buffer (10 mM Tris-HCl, one mM EDTA [pH 7.6]).

***Wolbachia* Screen.** Extracted DNA from individual beetles was screened for *Wolbachia* infection by PCR amplification of a 16S rRNA gene fragment. PCR was

Table 2. List of collection sites, dates, and species assayed shown in Fig. 1

Site	Location	Species Assayed[a]	Collection Date(s)
1	California-Alameda Co.	UUN	7-IV-99
2	New Mexico-Colfax Co.	LEM, UHO, VVI	13-VIII-98 25-VII-98
3	Colorado-Washington Co.	UHO	15-VIII-98
4	Colorado-Bent Co.	VVI	28-VIII-98
5	Nebraska-Dundy Co.	LON	13-VIII-98
6	Kansas-Wallace Co.	VVI	15-VIII-97
7	Texas-Hale Co.	VVI	?-VII-98
8	Kansas-Decatur Co.	BLA, UHO, VVI	16-VIII-97
9	Kansas-Scott Co.	LON	26-VII-98
10	Kansas-Finney Co.	VVI	26-VII-98
11	Nebraska-Webster Co.	BLA, LON	28-VII-98
12	Nebraska-Nuckolls Co.	LON, VIT, VVI	28-VII-98
13	Texas-Bell Co.	VZE	25-VI-98
14	Nebraska-Madison Co.	BAR	16-IX-98
15	South Dakota-Brookings Co.	BAR	13-IX-98
16	Nebraska-Dixon Co.	VVI	10-VIII-98
17	Nebraska-Lancaster Co (9-Mile prairie)	BAR, CRI	3-VIII-98 18-VIII-98
18	Nebraska-Lancaster Co. (Wulf Tall Grass prairie)	BAR, CRI, VIT	18-VIII-98
19	Minnesota-Mcleod Co.	BAR	31-VIII-99
20	Minnesota-Martin Co.	BAR	31-VIII-99
21	Iowa-Buena Vista Co.	BAR	31-VIII-99
22	Iowa-Clinton Co.	BAR	27-VII-98
23	Illinois-Champaign Co.	VVI	31-VII-98
24	Indiana-St. Joseph Co.	VVI	4-VIII-98
25	Alabama-Lee Co.	BAL	?-VIII-98
26	Florida-Indian River Co.	BAL	11-VIII-98
27	Brazil-Sete Largoas	SPE, VIR	?-VII-98
28	Panama-Cordillera	POR, VIR	25-VII-99

[a]Species codes are listed in Table 1.

conducted using *Wolbachia* specific primers 99 F (forward) (5′-TTGTAGCCTGCTATGGTATAACT-3′) and 994R (reverse) (5′-GAATAGGTATGATTTTCATGT-3′) (O'Neill et al. 1992). PCR was done in 25 μl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 300 μM dNTPs, 0.8 μM of each primer, 1.25

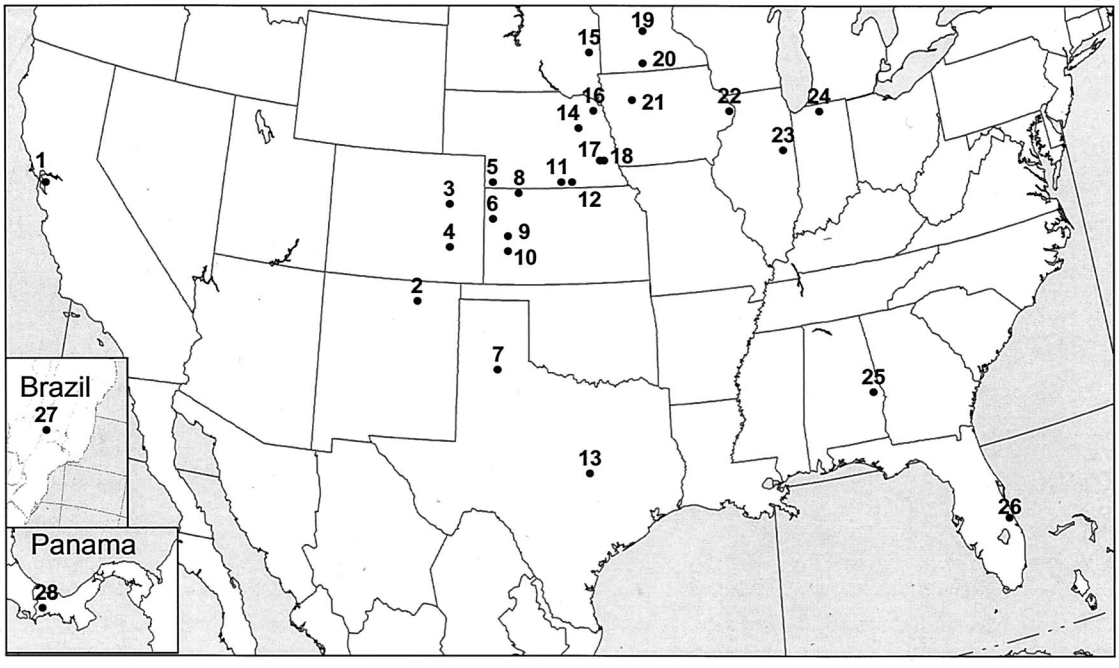

Fig. 1. Diabroticite collection sites.

U of Amplitaq polymerase (Perkin Elmer, Branchburg, NJ), 3.5 mM $MgCl_2$, and 3 μl of DNA template (diluted 1:10 from the original CTAB extraction). Thermocycling was then conducted on either a GeneAmp PCR system 2400 or 9600 (Perkin Elmer, Branchburg, NJ) thermocyclers with the following temperature profile: Hold at 95°C for 4 min; 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min; and a final extension step of 72°C for 7 min. All amplification groups had a negative control containing no DNA template. Because *D. v. virgifera* was already known to harbor *Wolbachia* (Giordano et al. 1997), we used this species as a positive control for all screening reactions.

***Wolbachia* Verification and Strain Identification.** Strain verification and identification was done using two methods. First, we amplified overlapping fragments of the 16S rRNA gene for two *Wolbachia* positive individuals from each species using the primer combinations of 21 F [5′-AYTTTGAGAGTTTGATCCTG-3′ (O'Neill et al. 1992)] and 994R, and 99 F and 1492R (5′GGTTACCTTGTTACGACTT-3′ (Giordano et al. 1995)] for DNA sequencing. PCR amplification of these fragments was done as described in the previous section. Amplified gene fragments were purified using a GenecleanII purification kit (Bio101, LaJolla, CA) and cloned directly into pCR 2.1 TOPO plasmid vector (TA cloning kit, Invitrogen, Carlsbad, CA) using the manufacturer's protocol. Positive clones were sequenced in both directions using a dye primer sequencing protocol at the University of Nebraska, DNA Sequencing Core Research Facility (Lincoln, NE) using a Li-Cor model 4000L DNA sequencer (Li-Cor, Lincoln, NE).

Second, we verified infection status and strain by PCR amplification (all positive individuals), restriction digestion (all positive individuals), and sequencing (single individuals from each positive species) of the bacterial cell-cycle gene, *fts*Z. PCR amplification was done using the primers *fts*Zf1 (5′-GTTGTCGCAAATACCGATGC-3′) and *fts*Zr1 (5′-CTTAAGTAAGCTGGTATATC-3′) (Werren et al. 1995a) in 25 μl with the same reaction ingredients and thermocycling profile as described for 16S rRNA.

Restriction digestion of *fts*Z amplicons was done with the restriction endonuclease *Dra*I that is diagnostic for many strains of type A *Wolbachia* including the strain known to infect *D. virgifera virgifera* (Giordano et al. 1997). Digests with *Dra*I were done in 15-μl reaction volumes containing 1.5 μl NEBuffer 4 (New England Biolabs, Beverly, MA), 6 μl PCR product, 5.75 μl of ddH_2O, and 0.5 μl (10U) of *Dra*I. The reaction mixtures were incubated at 37°C for 2 h. Following digestion *fts*Z amplicons were fractionated in 1% TAE agarose gels stained with ethidium bromide for visualization over a UV transilluminator. The *fts*Z amplicons were purified using a Geneclean II kit (Bio101) and directly sequenced in both directions using IRDye 800 dye terminator cycle sequencing kit (Li-Cor) following the manufacturers protocol on a Li-Cor IR^2 DNA Analyzer Gene Readir model 4200 (Li-Cor).

BLAST alignment searches using the BLASTN algorithm (BLASTN 2.0.13, NCBI Blast 2000) (Altschul et al. 1990) were done for both 16S rRNA and *fts*Z gene sequences to ascertain the identity of closely related sequences that are deposited in nonredundant GenBank, European Molecular Biology Laboratory (EMBL), DNA Data Bank of Japan (DDBJ), and Protein Data Bank (PDB). *Fts*Z sequences, along with selected sequences from GenBank, were also conceptually translated to amino acid sequences using GCG 10.1 for UNIX (Genetics Computer Group, Madison, WI) with the resulting amino acid sequences aligned using the Clustal W algorithm (Thompson et al. 1994) as implemented in GCG 10.1 using default parameters. After alignment of the sequences, distances were calculated on the Kimura 2-parameter (Kimura 1980) measure as implemented within GCG to ascertain the similarity of the sequences to one another as well as those selected from GenBank. These sequences were from two A division *Wolbachia* strains (accession nos. AB037896 [host: *Neochrysocharis formosa* (Westwood); Hymenoptera: Eulophidae] and WSP250969 [host: *Byturus tomentosus* (Scriba); Coleoptera: Byturidae], in addition to the the *D. v. virgifera* (accession no. AF011271) *fts*Z sequence published by Giordano et al. 1997, and two B division strains (accession nos. WSU28205 [host: *Nasonia vitripennis* (Walker), Hymenoptera: Pteromalidae] and AF011269 [host: *Gryllus integer* Scudder, Orthoptera: Gryllidae]). The resulting distance matrix was used to construct a similarity tree on the neighbor-joining algorithm (Saitou and Nei 1987) using the program GROWTREE as implemented within GCG 10.1. It was not the purpose of this analysis to propose a *Wolbachia* phylogeny but merely to show the similarity of the sequences to one another as well as some that are already published. All sequences are available from GenBank (Accession Nos. AY007547-AY007551 for 16S and AY007552-AY007556 for *fts*Z.

Results and Discussion

Three hundred thirty-one individuals representing 14 Diabroticite species and subspecies were screened by PCR assay using *Wolbachia* specific 16 rRNA gene primers. Five species (3 *Diabrotica* and two *Acalymma*) had individuals that were positive for *Wolbachia* infection. Three species [*D. lemniscata* LeConte, *D. v. virgifera*, and *A. blandulum* (LeConte)] had all individuals test positive for infection. Two species, *D. cristata* (Harris) and *A. vittatum* (F.), had three out of 25 and one out of 20 individuals test positive for infection, respectively.

Sequencing of 1,472 bp of the 16S rRNA gene from two positive individuals for each positive species (only one for *A. vittatum*) yielded only a few subtle sequence differences ranging from 0.00 to 0.27 substitutions per 100 bp. Although amplification and subsequent sequencing of the16S rRNA gene was adequate for the determination of *Wolbachia* infection status, the similarity of the sequence data led us to evaluation of the *fts*Z gene for identification pur-

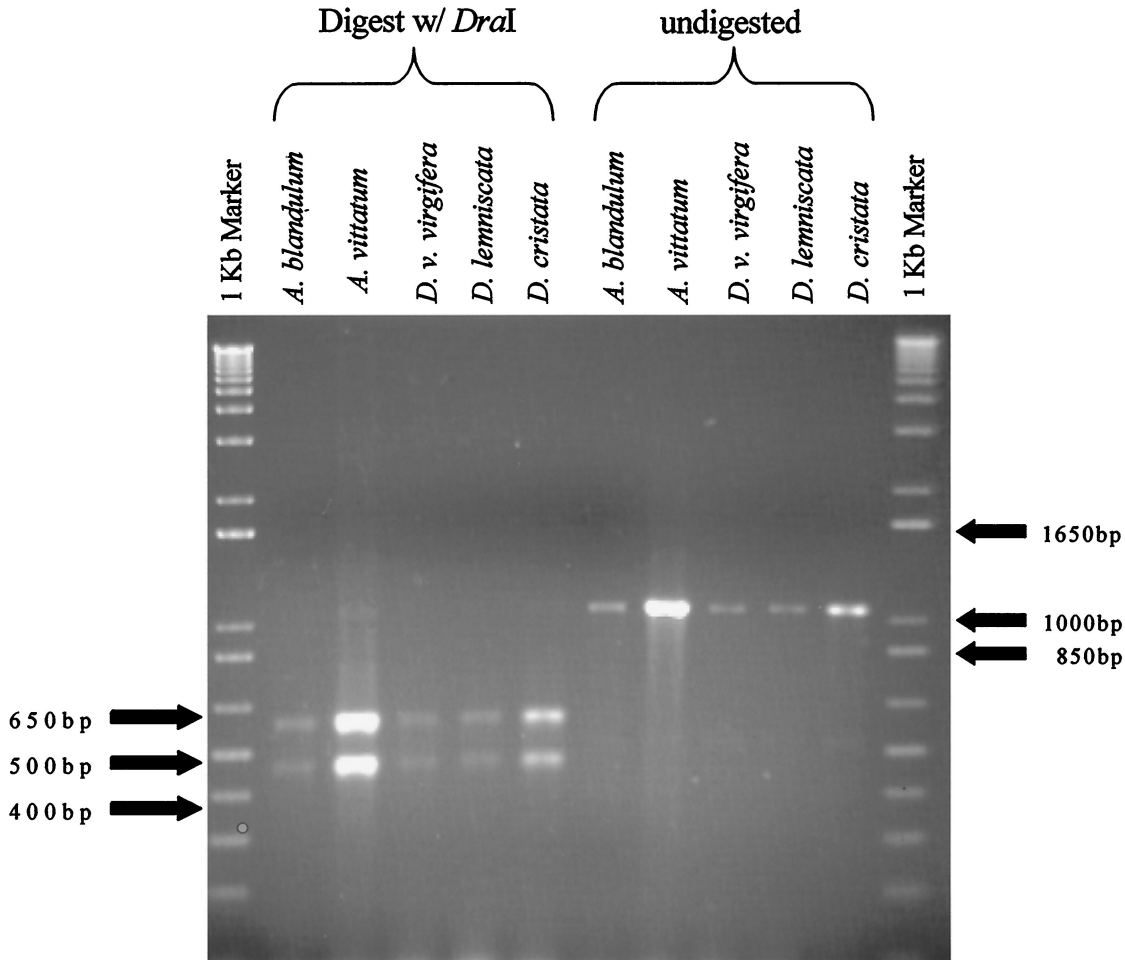

Fig. 2. Illustration of 1,045 bp *fts*Z PCR amplicon undigested and digested with the restriction endonuclease *Dra*I electrophoresed on 1.0% agarose gel stained with ethidium bromide.

poses as the *fts*Z gene has shown more resolving power than the 16S rRNA gene (Werren et al. 1995b, Sinkins and O'Neill 2000). Restriction digests of ≈1,045 bp *fts*Z amplicons with the diagnostic endonuclease, *Dra*I (Giordano et al. 1997), for all positive individuals yielded two fragments that were ≈ 585 and 460 bp respectively (Fig. 2). This result indicated that the *Wolbachia* strains infecting the Diabroticites most likely belong to division A, as our review of several published *fts*Z sequences within GenBank indicated that many A division *Wolbachia* contain the same *Dra*I restriction site.

We sequenced 891 bp (888 bp for *D. cristata*) of the ≈1,045 bp *fts*Z PCR amplicons. Alignment with the selected GenBank sequences used in this study resulted in analysis of 864 characters after pruning 27 bp on the 3′ end of the Diabroticite sequences that were not present in the GenBank sequences used. The Diabroticite species had Kimura 2-paramenter distances that ranged from 0.23 to 1.76 substitutions per 100 bases (Table 3). Interestingly, distances between *fts*Z data collected from *A. blandulum*, *A. vittatum*, *D. lemniscata*, and *D. v. virgifera* ranged from 0.23 to 0.35, while the sequence data from *D. cristata* compared with the other four Diabroticites ranged 1.64–1.76 substitutions per 100 bases (Table 3). Distances between Diabroticite *fts*Z and B division sequences ranged from 12.14 to 13.95 substitutions per 100 nucleotides, whereas Diabroticite comparisons with the selected A division strains ranged from 1.05 to 2.97 (Table 3). The neighbor-joining tree (Fig. 3) illustrates the similarity between A division *Wolbachia* sequenced in this study with data obtained from *A. blandulum*, *A. vittatum*, *D. lemniscata*, and *D. v. virgifera* forming a distinct cluster with branch lengths of minimal distance. Meanwhile, data from *D. cristata* clustered with the A strain sequences added from GenBank (Fig. 3). All A division *fts*Z sequences obtained from Diabroticites and those added from GenBank were distinctly distant from the B division sequences. Additionally, BlastN alignment searches of *fts*Z sequence data resulted in high alignment scores with hundreds of *Wolbachia* strains identified as belonging to division A. The best BlastN alignment

Table 3. Pairwise *ftsZ* distance matrix for the estimated number of substitutions per 100 bases corrected by the Kimura 2-parameter method

	1	2	3	4	5	6	7	8	9	10
1. *D. v. virgifera*	–	0.35	0.35	0.35	0.23	1.76	2.12	2.97	13.44	12.42
2. *A. blandulum*		–	0.23	0.35	0.23	1.76	2.12	2.97	13.44	12.42
3. *D. lemniscata*			–	0.23	0.12	1.64	2.00	2.73	13.29	12.27
4. *A. vittatum*				–	0.12	1.65	2.00	2.85	13.30	12.28
5. *D. v. virgifera*[a]					–	1.53	1.88	2.73	13.16	12.14
6. *D. cristata*						–	1.05	2.36	13.66	12.63
7. *Neochrysocaris formosa*[a]							–	2.00	13.50	12.48
8. *Byturus tomentosus*[a]								–	13.95	13.21
9. *Gryllus integer*[a]									–	1.43
10. *Nasonia vitripennis*[a]										–

[a]Species previously published in GenBank.

scores for data from *A. blandulum*, *A. vittatum*, *D. lemniscata*, and *D. v. virgifera* were with a previously published *fts*Z sequence by Giordano et al. (1997) from *D. v. virgifera.* Meanwhile, the best BlastN alignment score for *fts*Z data collected from *D. cristata* was with a *Wolbachia* strain isolated from *N. formosa.* On the basis of the distance matrix data, the neighbor joining tree, and BlastN alignment searches, we conclude that the strain of *Wolbachia* infecting *A. blandulum*, *A. vittatum*, *D. lemniscata*, and *D. v. virgifera* are very similar (possibly the same), but different from the strain infecting *D. cristata.* According to Giordano et al. (1997), the strain infecting *D. v. virgifera* is a basal strain or species in the A division of *Wolbachia.* However, a formal name has not been given to these *Wolbachia* as the nomenclature of these endosymbionts is in a state of taxonomic flux (Werren 1997).

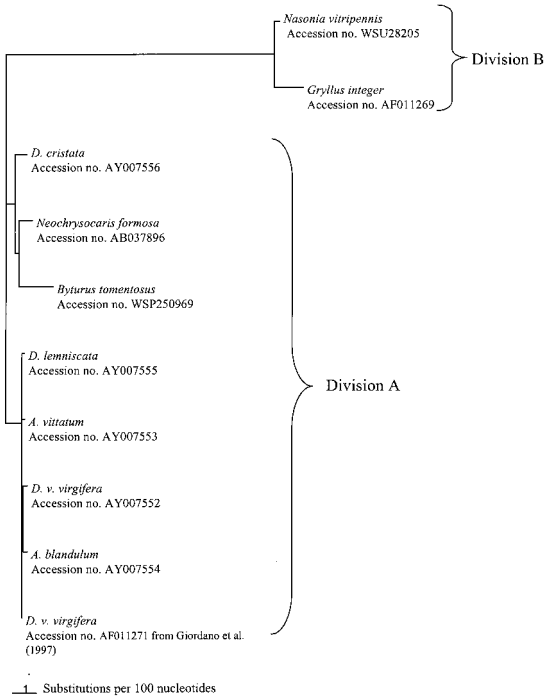

Fig. 3. Neighbor-joining GROWTREE phylogram based on analysis of *fstZ* sequences using Kimura's 2-parameter correction method. Branch lengths are proportional to inferred substitutions per 100 bp.

Because we observed a similar same strain of *Wolbachia* infecting all positive individuals of *D. v. virgifera*, *D. lemniscata*, *A. blandulum*, and *A. vittatum*, it is possible that infections were horizontally transferred between these Diabroticite species. Horizontal transmission of *Wolbachia* is thought to be a common phenomenon between insect species although it may occur infrequently. For example, among anopheline mosquitos, *Aedes*, *Culex*, and *Mansonia* species harbor high rates of *Wolbachia* infection, while infection is conspicuously absent in *Anopheles* species (Kittayapong et al. 2000). The potential routes of horizontal transmission or exchange between species are currently unknown, however, it has been suggested that predators, parasites, prey, and associated competition are potential routes of horizontal transmission (Werren 1997). The Diabroticites surveyed in this study have a common trait that can potentially bring many species together in a temporal and spatial fashion. The trait is an affinity toward triterpene cucurbitacins found in the plant family Cucurbitaceae (Metcalf 1979, Metcalf et al. 1980), mediated by $GABA_A$/glycine taste receptors (Mullin et al. 1994). Because of this affinity, it is common to observe several Diabroticites on cucurbits such as *Cucurbita foetidissima* HBK. For example, like other authors, we have observed several species using the same *C. foetidissima* plant or even the same bloom numerous times in the field (Golden 1990; T.L.C. and L.J.M., unpublished data). Thus, a close association with cucurbits may provide a potential arena for horizontal transmission of *Wolbachia*, although it would most likely be infrequent based on the absence of infection in many of the species assayed.

In their study on *Wolbachia* infection of *D. virgifera* spp., Giordano et al. (1997) proposed that *Wolbachia* infection for this species most likely originated in northwestern Mexico or the southwestern United States (New Mexico and western Texas). This conclusion is plausible based on the geographic ranges of the *Wolbachia* positive *D. v. virgifera* and *Wolbachia* negative *D. v. zeae* Krysan & Smith (Krysan and Smith

1987). Our results neither confirm nor deny this hypothesis as all species we found to be *Wolbachia* positive also occur in northwestern Mexico and the southwestern United States. However, two species, *D. longicornis* and *D. u. howardi*, also occur in this geographic range yet were negative for *Wolbachia* infection. It is also interesting to note that no species that occurs outside the before mentioned geographic area tested positive for infection although *D. longicornis* was not collected south of Kansas for this analysis. A more systematic survey of Diabroticite populations, particularly those that tested positive for infection, may provide insight to the geographic origins of *Wolbachia* infections within Diabroticites. Also, it may be more valuable to focus such a survey on species, like *D. lemniscata* or *A. blandulum*, that are not influenced by agriculture, as geographic range of some Diabroticites can be greatly influenced by widespread monoculture of a preferred larval host. The rapid expansion of the geographic range of *D. v. virgifera* across maize production regions in North America is a classic example of Diabroticite expansion (Krysan and Smith 1987).

While *Wolbachia* has been implicated as a factor influencing rapid speciation between populations (Coyne 1992), our results imply that the observed unidirectional reproductive incompatibility between the morphological sibling species, *D. barberi* and *D. longicornis*, is most likely due to some other mechanism. Although it cannot be ruled out that *Wolbachia* was the original barrier, with infections being secondarily lost over time. While *Wolbachia* induced separation between *D. barberi* and *D. longicornis* is unlikely, Giordano et al. (1997) in their reproductive incompatibility study between the subspecies *D. v. virgifera* and *D. v. zeae* provided preliminary evidence that a speciation event may be occurring. This is further indicated by fairly distinct geographic demarcation zones in the Texas panhandle and Mexico despite potentially favorable habitats for both subspecies on either side of current geographic boundaries (Krysan and Smith 1987). In contrast, *D. barberi* and *D. longicornis* have a relatively large area of sympatry that covers a large area of Kansas and Nebraska (Krysan and Smith 1987). Furthermore, genetic distance data obtained from 1,323 bp of the mitochondrial cytochrome oxidase subunit one (COI) gene revealed a distance between *D. v. virgifera* and *D. v. zeae* of 0.0472, whereas the distance between *D. barberi* and *D. longicornis* was 0.0113 (Clark et al. 2001). These results indicate that *Wolbachia* infections may create a more distinct gene flow barrier between populations or closely related species than the current mechanism which is driving the separation of morphological sibling species, such as *D. barberi* and *D. longicornis*, as evidenced by COI genetic distance data.

Regarding *D. cristata*, it was interesting to observe both *Wolbachia* positive and negative individuals within the same populations. *D. cristata* is a univoltine species that is closely tied to relict prairie ecosystems where it has been found to be associated with *Andropogon gerardi* Vitman (Yaro and Krysan 1986, Krysan and Smith 1987). This species is usually collected as adults on a variety of prairie forbs and is occasionally collected on maize and *C. foetidissima* if either plant is near a remnant prairie (T.L.C. and L.J.M., unpublished data). The low frequency of *Wolbachia* infection within the *D. cristata* populations surveyed (three out of 25 individuals) suggests a few possibilities in terms of *Wolbachia* infection acquisition. The first possibility is that *Wolbachia* has been in this species for a long period of time with no impact on reproductive incompatibility selection characteristics, such as an increased infection rate or a genetic bottleneck (Turelli et al. 1992, Rousset and Solignac 1995). However, this relationship will not be known until more extensive surveys take place coupled with incompatibility studies. Another possibility is that infections were recently acquired from a parasite as various *Wolbachia* strains have been detected in many parasitic wasp species (Werren 1997). A third possibility is that infections were transferred horizontally from *D. v. virgifera* populations because these species have recently become sympatric in specific areas over the last 50 yr. The region where our *D. cristata* collections were obtained is within the range of *D. v. virgifera* expansion across large-scale maize production regions throughout the north central United States. However, differences between *fts*Z sequences suggest that horizontal transfer from *D. v. virgifera* is probably the most unlikely scenario.

If the *Wolbachia* strain infecting *D. cristata* causes cytoplasmic incompatibility, as has been observed in *D. v. virgifera* (Giordano et al. 1997), then we would expect *D. cristata* to eventually have reduced mitochondrial DNA variation as *Wolbachia* infection is maternally inherited like mitochondrial DNA (Turelli et al. 1992, Rousset and Solignac 1995) as well as a higher incidence of infection given the reproductive advantage of *Wolbachia* induced individuals. However, if the infection was recent, it would take several years to see high levels of infection as well as restricted mitochondrial variation. For example, Turelli and Hoffmann (1991) reported that it took 15 generations for *Drosophila simulans* to go from an infection frequency of 30–80% in some California locations. Such an increase would take at least 15 yr for *D. cristata*, given its univoltine life cycle (Krysan 1982), without considering, for instance, potential alterations in reproductive biology or mating behavior that could be altered by *Wolbachia*. In terms of mitochondrial DNA variation, we have observed three mitochondrial haplotypes for *D. cristata* versus only one haplotype each for *D. v. virgifera* and *D. lemniscata* populations when cutting a 1,308 bp portion of the mitochondrial COI with restriction enzymes (Clark 2000), which is another indicator of recent infection status for *D. cristata*. It would be of value to conduct a large-scale survey of *Wolbachia* infections within *D. cristata* as well as a series of reproductive biology studies on this species to clarify our observations.

In summary, our data show that *Wolbachia* infections are present in Diabroticite species beyond *D. v. virgifera*. It remains unclear whether these infections

are responsible for population or species isolation, although it cannot be ruled out that *Wolbachia* may have been an original barrier between closely related species like *D. barberi* and *D. longicornis* as infections may have been secondarily lost over time. Because our results do indicate that many Diabroticites are able to harbor *Wolbachia* infection, it may be possible to introduce infections to uninfected pest species that could have beneficial applications as it has been suggested that *Wolbachia*-induced cytoplasmic incompatibility may have the potential to drive beneficial genes within pest populations (Sinkins and O'Neill 2000). For example, it may be possible to introduce and spread genes that inhibit or eliminate the transfer of plant pathogens, such as bacteria or viruses, or possibly reintroduce insecticide susceptibility in a given insect population. However, studies pertaining to the acquisition of *Wolbachia* infection for noninfected individuals via techniques like microinjection (Clancy and Hoffmann 1997) or hemolymph transfer (Grenier et al. 1998) would be a necessary starting point. Future studies in this area may have application within the Diabroticite group, as several species within the genera *Diabrotica* and *Acalymma* are notorious for their ability to transmit plant diseases (Gergerich et al. 1986, Latin 1993) and develop resistance to a variety of insecticides (Ball and Weekman 1963, Meinke et al. 1998).

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References Cited

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–410.

Ball, H. J., and G. T. Weekman. 1963. Differential resistance of corn rootworms to insecticides in Nebraska and adjoining states. J. Econ. Entomol. 56: 553–555.

Black IV, W. C., and N. M. DuTeau. 1997. RAPD-PCR and SSCP analysis for insect population genetic studies, pp. 361–373. *In* J. M. Crampton, C. B. Beard, and C. Louis [eds.], The molecular biology of insect disease vectors: a methods manual. Chapman & Hall.

Bouchon, D., T. Rigaud, and P. Juchault. 1998. Evidence for widespread *Wolbachia* infection in Isopod crustaceans: molecular identification and host feminization. Proc. R. Soc. Lond. Ser. B Biol. Sci. 265: 1081–1090.

Clancy, D. J., and A. A. Hoffmann. 1997. Behavior of *Wolbachia* from *Drosophila simulans* in *Drosophila serrata,* a novel host. Am. Nat. 149: 975–988.

Clark, T. L. 2000. Marker based diagnosis, molecular phylogenetics and discovery of population altering endosymbionts for selected Diabroticites (Coleoptera: Chrysomelidae). Ph.D. dissertation. University of Nebraska-Lincoln.

Clark, T. L., L. J. Meinke, and J. E. Foster. 2001. Molecular phylogeny of *Diabrotica* beetles (Coleoptera: Chrysomelidae) inferred from analysis of combined mitochondrial and nuclear DNA sequences. Insect Mol. Biol. 10: 303–314.

Coyne, J. A. 1992. Genetics and speciation. Nature (Lond.) 355: 511–515.

Degrugillier, M. E., S. S. Degrugillier, and J. J. Jackson. 1991. Nonoccluded, cytoplasmic virus particles and Rickettsia-like organisms in testes and spermathecae of *Diabrotica virgifera.* J. Invertebr. Pathol. 57: 50–58.

Gergerich, R. C., H. A. Scott, and J. P. Fulton. 1986. Evaluation of *Diabrotica* beetles as vectors of plant viruses, pp. 227–249. *In* J. L. Krysan and T. A. Miller (eds.), Methods for the study of pest *Diabrotica,* Springer, New York.

Giordano, R., S. L. O'Neill, and H. M. Robertson. 1995. Wolbachia infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana.* Genetics 140: 1307–1310.

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

Golden, K. L. 1990. Cuticular hydrocarbons of sibling species *Diabrotica longicornis* (Say) and *D. barberi* Smith & Lawrence: use as chemotaxonomic characters for species separation and study of population variation. M.S. thesis, University of Nebraska-Lincoln.

Grenier, S., B. Pintureau, A. Heddi, F. Lassabliere, C. Jager, C. Louis, and C. Khatchadourian. 1998. Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps. Proc. R. Soc. Lond. Ser. B Biol. Sci. 265: 1441–1445.

Hoffmann, A. A., M. Hercus, and H. Dagher. 1998. Population dynamics of the *Wolbachia* infection causing cytoplasmic incompatibility in *Drosophila melanogaster.* Genetics 148: 221–231.

Hoffmann, A. A., and M. Turelli. 1997. Cytoplasmic incompatibility in insects, pp. 42–80. *In* S. L. O'Neill, A. A. Hoffmann, and J. H. Werren [eds.], Influential passengers: inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparitive studies of nucleotide sequences. J. Mol. Evol. 16: 111–120.

Kittayapong, P., K. J. Baisley, V. Baimai, and S. L. O'Neill. 2000. Distribution and diversity of *Wolbachia* infections in southeast Asian mosquitoes (Diptera: Culicidae). J. Med. Entomol. 37: 340–345.

Krysan, J. L. 1982. Diapause in the nearctic species of the *virgifera* group of *Diabrotica:* evidence for tropical orgin and temperate adaptations. Ann. Entomol. Soc. Am. 75: 136–142.

Krysan, J. L. 1986. Introduction: biology, distribution, and identification of pest *Diabrotica,* pp. 1–23. *In* J. L. Krysan and T. A. Miller [eds.], Methods for the study of pest *Diabrotica.* Springer, New York.

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

Krysan, J. L., R. F. Smith, T. F. Branson, and P. L. Guss. 1980. A new subspecies of *Diabrotica virgifera* (Coleoptera:

Chrysomelidae): description, distribution, and sexual compatibility. Ann. Entomol. Soc. Am. 73: 123–130.

Krysan, J. L., R. F. Smith, and P. L. Guss. 1983. *Diabrotica barberi* (Coleoptera: Chrysomelidae) elevated to species rank based on behavior, habitat choice, morphometrics, and geographical variation of color. Ann. Entomol. Soc. Am. 76: 197–204.

Latin, R. X. 1993. Bacterial wilt, p. 29. *In* R. X. Latin [ed.], Diseases and pests of muskmelons and watermelons. Purdue University, Cooperative Extension Service, West Lafayette, IN.

Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. J. Econ. Entomol. 91: 594–600.

Metcalf, C. L., W. P. Flint, and R. L. Flint. 1962. Destructive and useful insects, 4th ed. McGraw-Hill, New York.

Metcalf, R. L. 1979. Plants, chemicals, and insects: some aspects of coevolution. Bull. Entomol. Soc. Am. 25: 30–35.

Metcalf, R. L. 1986. Forward, pp. vii-xv. *In* J. L. Krysan and T. A. Miller (eds.), Methods for the study of pest *Diabrotica.* Springer, New York.

Metcalf, R. L., R. A. Metcalf, and A. M. Rhodes. 1980. Cucurbitacins as kairomones for diabroticite beetles. Proc. Natl. Acad. Sci. U.S.A. 77: 3769–3772.

Mullin, C. A., S. Chyb, H. Eichenseer, B. Hollister, and J. L. Frazier. 1994. Neuroreceptor mechanisms in insect gustation: a pharmological approach. J. Insect Physiol. 40: 913–931.

Munroe, D. D., and R. F. Smith. 1980. A revision of the systematics of *Acalymma sensu stricto* Barber (Coleoptera: Chrysomelidae) from North America including Mexico. Mem. Entomol. Soc. Can. 112: 1–92.

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.

Perrot-Minnot, M.-J., L. R. Guo, and J. H. Werren. 1996. Single and double infections with *Wolbachia* in the parasitic wasp *Nasonia vitripennis:* effects on compatibility. Genetics 143: 961–972.

Rousset, F., and M. Solignac. 1995. Evolution of single and double *Wolbachia* symbiosis during speciation in the *Drosophila simulans* complex. Proc. Natl. Acad. Sci. U.S.A. 92: 6389–6393.

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

Sinkins, S. P., and S. L. O'Neill. 2000. *Wolbachia* as a vehicle to modify insect populations, pp. 271–287. *In* A. M. Handler and A. A. James [eds.], Insect transgenesis: methods and applications. CRC, Boca Raton, FL.

Smith, R. F. 1966. Distributional patterns of selected western North American insects: The distribution of Diabroticites in western North America. Bull. Entomol. Soc. Am. 12: 108–110.

Smith, R. F., and J. F. Lawrence. 1967. Clarification of the type specimens of Diabroticites (Coleoptera, Chrysomelidae, Galerucinae). Univ. Calif. Publ. Entomol. 45: 1–174.

Stouthamer, R., J.A.J. Breeuwer, R. F. Luck, and J. H. Werren. 1993. Molecular identification of microorganisms associated with parthenogenesis. Nature (Lond.) 361: 66–68.

Thompson, J. D., D. G. Higgins, and T. J. Higgins. 1994. Clustal W: Improving the sensitivity of progressive sequence alignment through sequence weighting, position gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

Turelli, M., and A. A. Hoffmann. 1991. Rapid spread of an inherited incompatibility factor in California *Drosophila.* Nature (Lond.) 353: 440–442.

Turelli, M., A. A. Hoffmann, and S. W. McKechnie. 1992. Dynamics of cytoplasmic incompatibility and mtDNA variation in natural *Drosophila 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. R. Guo. 1995a. Distribution of *Wolbachia* among neotropical arthropods. Proc. R. Soc. Lond. Ser. B Biol. Sci. 261: 55–63.

Werren, J. H., W. Zhang, and L. R. Guo. 1995b. Evolution and phylogeny of *Wolbachia*–reproductive parasites of arthropods. Proc. R. Soc. Lond. B Biol. Sci. 261: 55–63.

Yaro N. and J. L. Krysan. 1986. Host relationships of *Diabrotica cristata* (Coleoptera: Chrysomelidae). Entomol. News 97: 11–16.

Yen, J. H., and A. R. Barr. 1973. The etiological agent of cytoplasmic incompatibility in *Culex pipiens.* J. Invertebr. Pathol. 22: 242–250.

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