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S. J. Erney
University of Nebraska-Lincoln

K. P. Pruess
University of Nebraska-Lincoln

S. D. Danielson
University of Nebraska-Lincoln

T. O. Powers
University of Nebraska-Lincoln, tpowers1@unl.edu

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Molecular Differentiation of Alfalfa Weevil Strains (Coleoptera: Curculionidae)

S. J. Erney,[1] K. P. Pruess,[1] S. D. Danielson,[1] and T. O. Powers[2]

1. Department of Entomology, University of Nebraska–Lincoln, Lincoln, Nebraska, USA
2. Department of Plant Pathology, University of Nebraska–Lincoln, Lincoln, Nebraska, USA

Abstract
Mitochondrial DNA was amplified and sequenced from eastern, western, and Egyptian strains of alfalfa weevil, *Hypera postica* (Gyllenhal). Eastern and Egyptian weevils differed at only 2 nucleotide sites in 1,031 base pairs sequenced; western weevils differed by 5% sequence divergence. Three restriction sites were identified which separated eastern and western haplotypes. No intrastrain polymorphism was detected in 150 weevils from Nebraska. Collections from Lincoln in eastern Nebraska and Scottsbluff in western Nebraska were fixed for the eastern and western haplotypes, respectively. Eastern and western haplotypes were found together in the same fields in a broad overlap region in central Nebraska.

Keywords: *Hypera postica*, alfalfa weevil strains, mtDNA, PCR-RFLP, DNA sequence

Alfalfa weevil, *Hypera postica* (Gyllenhal), is thought to have been introduced into the United States from Europe or Asia on three separate occasions (Woods et al. 1978). The western strain was first reported in the United States in 1904 near Salt Lake City, Utah. The second introduction, referred to as the Egyptian alfalfa weevil and formerly considered a separate species, *Hypera brunneipennis* (Boheman), was first reported near Yuma, Arizona, in 1939. The third introduction, the eastern strain, was first reported from Maryland in 1952. These strains spread rapidly, and the alfalfa weevil is now considered to be a major economic pest of alfalfa, *Medicago sativa* L., in all 48 contiguous states.

Klostermeyer and Manglitz (1979) suggested that these three strains have been in contact since the early 1970s with the potential for hybridization between eastern and western

weevils across a broad zone in the Midwest. Hsiao (1993) found Egyptian and western weevils occurring sympatrically in five states west of the Rocky Mountains.

Morphological characters used to distinguish strains (Pienkowski et al. 1969, Bland 1984) were based on field-collected adults, and environmental effects were not considered in the derivation of these characters. Although individual weevils cannot be differentiated morphologically, there exist physiological, behavioral, and ecological differences among strains which influence their economic impact and management. Eastern and Egyptian weevils are reported to have a greater ability to encapsulate eggs of a primary parasite, *Bathyplectes curculionis* (Thomsom) (Puttier 1967), but Manglitz et al. (1981) found a higher rate of parasitism in the eastern strain in Nebraska. In areas where significant parasitism occurs, management practices have been designed to protect the parasite. Consequences of mixing strains are unknown, and were a major concern in parasite releases where unparasitized weevils might be released into an area occupied by another strain (Kingsley et al. 1993).

Egyptian and eastern weevils have summer and winter adult diapause with an intervening period of fall activity, which may result in both fall and spring oviposition in the South. The western strain has an obligate adult diapause without fall activity and only spring oviposition. Eastern weevils enter fields earlier in the spring in Nebraska (Manglitz et al. 1981, Kingsley et al. 1993). Egyptian weevils tend to pupate on plants, whereas eastern weevils normally pupate among debris on the soil surface (Salt and van den Bosch 1967).

Sell et al. (1978) surveyed allelic variation at five enzyme loci in eastern (7 populations) and western (3 populations) weevil adults. They found differences in gene frequencies between the two strains but no fully diagnostic loci. Hsiao and Stutz (1985) found 1 locus of alcohol dehydrogenase to be diagnostic for adult female eastern and western weevils but not for eastern and Egyptian or for males, in which it was absent.

Hsiao and Hsiao (1984) were unable to find karyotypic characters supporting species status of the Egyptian weevil, but Hsiao and Hsiao (1985) detected a maternally transmitted *Rickettsia* that caused cytoplasmic incompatibility between the eastern and western strains. Crosses between eastern females and western males result in inviable eggs, whereas the reciprocal cross yields viable eggs with a skewed sex ratio of 6:1 (females/males) (Blickenstaff 1965). Cross-mating experiments by Klostermeyer and Manglitz (1979) revealed a broad zone of apparent genetic incompatibility in Nebraska.

Hsiao (1996) sequenced 1,302 base pairs of the mitochondrial DNA genome of five weevil strains and found an average of 2% sequence divergence. He concluded that this was similar to intraspecific variation in other beetles, supporting the hypothesis that all weevil strains belong to the same species.

None of these studies can readily distinguish among weevil strains in all life stages. Positive identification may be important in developing pest management practices in different geographical areas. The objectives of this study were to identify strain-diagnostic mitochondrial markers and to assess the distribution of each strain in Nebraska based upon female ancestry.

Materials and Methods

Weevils

Alfalfa weevil larvae were collected in 1990 or 1991 by cooperators from near the three putative origins in Utah, Maryland, and Arizona and shipped to our laboratory alive. Larvae were collected from 11 locations in Nebraska in May 1991. Individual weevils were stored in microfuge tubes with 100 μl TE buffer (pH 7.5) at –70°C.

DNA Analyses

DNA was isolated from individual larvae after removal of their digestive tracts. Weevils were homogenized in buffer containing proteinase K, digested 4 hr to overnight, and the extracts were subjected to RNAse or amylase digestions. DNA was extracted by standard phenol-chloroform methods (Sambrook et al. 1989), ethanol precipitated, resuspended in TE, and stored at –20°C until used.

Double-stranded PCR was done according to standard methods with amplifications using ≈5–10 ng genomic DNA in 25-μl total volumes. Five of the 6 primers used (C1-J-2797, CB-J-11545, Nl-N-11841, Nl-J-12585, LR-N-12854) were described by Simon et al. (1994). The 6th primer, C2-N-3686 (5′ CAATTGGTATAAAACTATG-ATTTGC 3′), was used in conjunction with Cl-J-2797. Amplicons are reported using 2-letter codes for annealing sites, e.g., C1/C2. Primers C1/C2 amplified portions of CO-I, CO-II, and intervening $tRNA^{Leu(UUR)}$ genes; CB/N1-N amplified portions of cytochrome b, ND1, and intervening $tRNA^{Ser(UCN)}$; and Nl-J/LR amplified portions of ND1, 16S rRNA, and intervening $tRNA^{Leu(CUN)}$. One additional primer was used in sequencing the C1/C2 amplicon: C2-N- 3380 (5′ TCAA TATCATTGATGACCAAT 3′).

Amplification conditions were denaturing at 94°C (1 min), annealing at 42°C (1 min), and extension at 72°C (1 min) for 35 cycles. Amplified PCR products were purified through Amicon C-100 microconcentrators (Amicon, Beverly, Massachusetts).

Two sequencing methods were used. For direct sequencing, primers were end-labeled using T4 polynucleotide kinase (Promega, Madison, Wisconsin) and γ^{32}P and used with Promega's fmol sequencing system according to manufacturer's recommendations. Alternatively, cloned products were sequenced. Amplified products were purified with Gene Clean II (Bio 101, La Jolla, California), end-repaired with T4 DNA polymerase (Promega), precipitated in ethanol, and products were ligated into *Sma*-cut pBlueScript II SK+ vector (Stratagene, La Jolla, California). Ligations were transformed into Stratagene *E. coli* XLl-Blue supercompetent cells and plated on selective media with blue-white color selection antibiotics. Individual white colonies were screened for proper size insert using initial PCR amplification conditions, except the number of cycles was reduced to 23. Colonies of successfully ligated clones were grown to single-strand DNA by VCS M13 Helper Phage (Stratagene) and sequenced with ^{35}S using Sequenase kit version 2.0 (U.S. Biochemical, Cleveland, Ohio).

DNA sequences were aligned by eye with the *Drosophila yakuba* Burla sequence (Clary and Wolstenholme 1985). Percentage sequence divergence was computed from the num-

ber of aligned nucleotides having different bases divided by the number of sequence positions compared. Sequences were searched (GCG, Wisconsin Genetics Computer Group, Madison, Wisconsin) for potentially diagnostic restriction enzyme recognition sites.

Restriction digests were performed using 10 units restriction enzyme (Promega), 1 μl appropriate restriction buffer, 3–6 μl amplified DNA (to provide similar total DNA), and sterile H_2O for a total volume of 10 μl. Digestions were done overnight at 37°C. Restriction products were routinely visualized by electrophoresis on 1.4% agarose gels stained with ethidium bromide, but for clarity of illustration of small fragments, 2.5% Metaphor agarose (FMC, Rockland, Maine) was used to prepare Figures 5 and 6.

Results and Discussion

Eastern and Egyptian weevils differed in only 2 nucleotides over all 1,031 base pairs (bp) of 3 mitochondrial DNA amplicons sequenced. These amplicons included portions of 4 protein genes, 3 complete tRNA genes, and a portion of the large ribosomal gene. Sequence differences between western and eastern (or Egyptian) weevils were found in all 3 amplicons.

Amplicon C1/C2

Direct sequence of amplicon C1/C2-N-3686 generated 478 bp which included partial sequences of CO-I, CO-II, and the complete intervening $tRNA^{Leu(UUR)}$ (Fig. 1).

The CO-I gene in weevils had 1 additional codon at the 3′ end relative to *D. yakuba*. Eastern and western weevils differed by 6 silent transitions in CO-I. Weevil strains differed by a single transition in the TψC loop of $tRNA^{Leu(UUR)}$.

The initiation codon for CO-II was ATC in eastern and Egyptian haplotypes, ATT in western ones. Liu and Beckenbach (1992) also reported ATT as the CO-II initiation codon in 2 other coleopterans sequenced. Of the 12 nucleotide differences detected in CO-II, 10 were silent transitions. The 2 transversions resulted from a substitution of serine codons (TCT versus AGT, positions 460–462; Fig. 1).

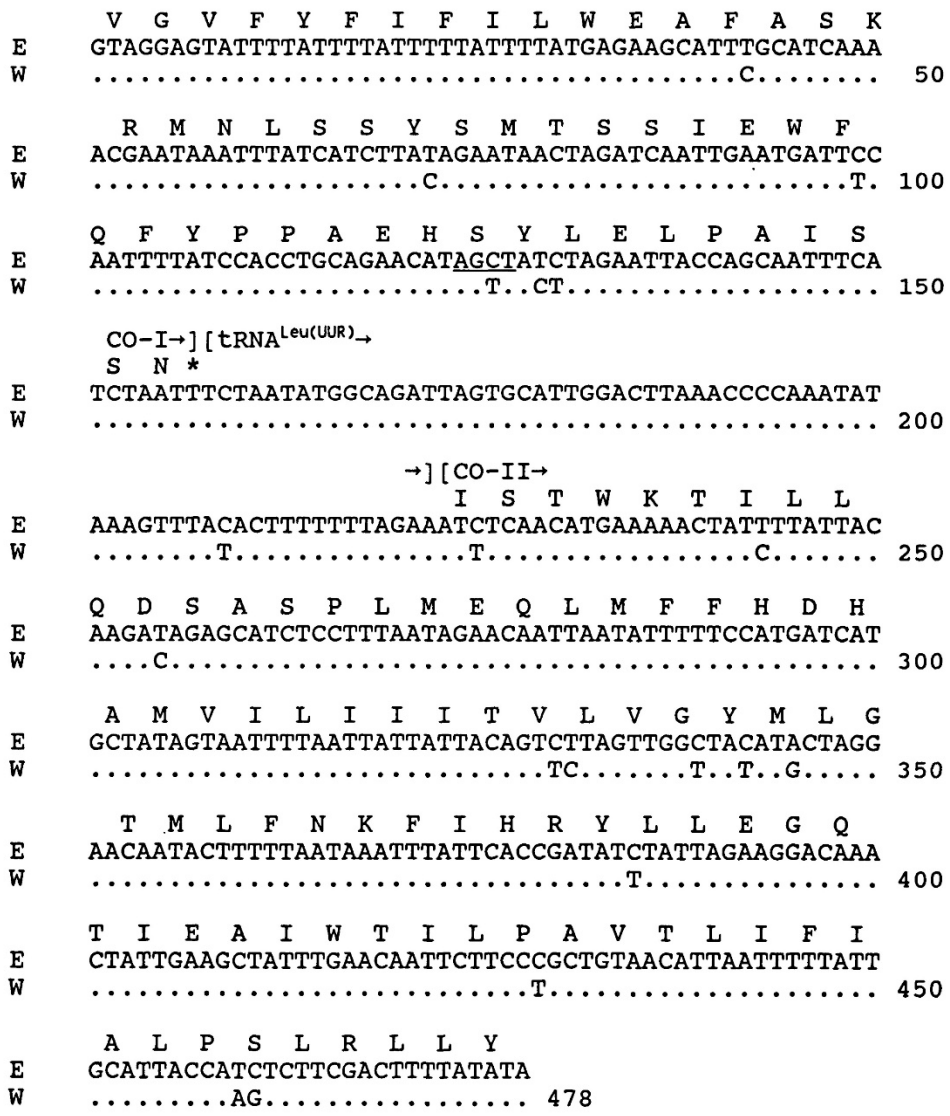

Figure 1. Partial sequence for C1/C2 amplicon obtained with primers Cl-J-2797 and C2-N-3380 for eastern and western strains of alfalfa weevil. Dots indicate identity. Egyptian strain was identical to eastern and is not shown. Sequence corresponds to nucleotides 2854–3338 in *D. yakuba* genome. Amino acid translation is shown by 1-letter codes. A potentially diagnostic *Alu*I restriction site is underlined.

Amplicon CB/N1

This amplicon produced 300 bp of comparative sequence and included portions of the Cytochrome b and ND1 genes and the complete intervening $tRNA^{ser(UCN)}$ gene (Fig. 2). Structural comparison with *D. yakuba* shows an inferred insertion of 2 codons at the 3′ end of the cytochrome b gene and a putative TA termination. A larger structural difference between alfalfa weevils and *D. yakuba* exists in the terminal region of ND1. Termination of

ND1, which is encoded on the minority strand, is inferred to be TAG at position 198 in weevils. This putative termination results in a deletion of 12 codons relative to *D. yakuba* and creates an intergenic region more similar to that in the honey bee (Crozier and Crozier 1993), which is 14 codons shorter than *D. yakuba*.

Among weevils strains there were 5 silent transitions and 2 transversions in cytochrome b. All strains differed in nucleotides 161 and 162 in the TψC loop in tRNA$^{Ser(UCN)}$ (Fig. 2). These were the only differences found between eastern and Egyptian weevils. In a partial direct sequence, we found a 2nd variant (CT) in eastern weevils at these positions, which represented the only intrastrain variation detected. This haplotype was reported by Hsiao (1996).

Weevil strains differed in 10 nucleotides in ND1, of which 9 were transitions but which created 4 conservative amino acid replacements. Three of these replacements occurred at the terminal 3′ end. This was the only gene in which amino acids differed between eastern and western weevils.

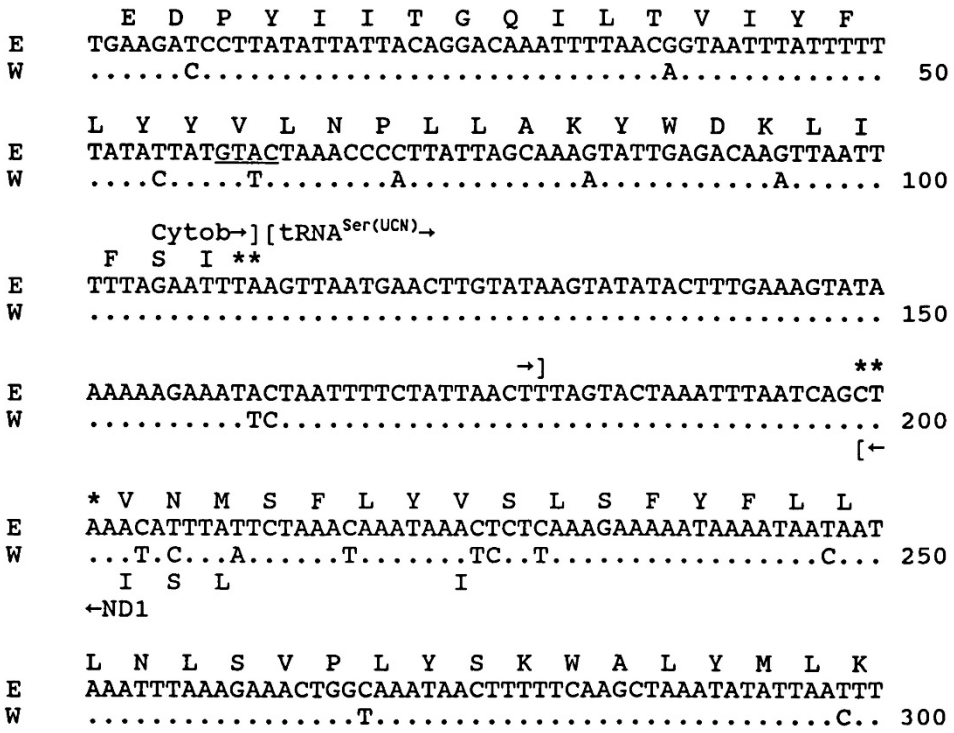

Figure 2. Sequence of CB/N1 amplicon obtained with primers CB-J-11545 and Nl-N-11841 for eastern and western alfalfa weevil strains. Dots indicate identity. Egyptian strain differed from eastern only in nucleotides 161 and 162 (TT) and is not shown. Sequence corresponds to nucleotides 11546–11840 in *D. yakuba*. Amino acid translation is shown above the sequence for eastern, below for western when different. A potentially diagnostic *Rsa*I restriction site is underlined.

Amplicon N1/LR

This amplicon produced 253 bp of comparative sequence and included portions of the ND1 and large ribosomal genes and the complete intervening tRNA$^{Leu(CUN)}$ gene. ND1 has 2 additional codons relative to *D. yakuba* at the 5′ end (Fig. 3).

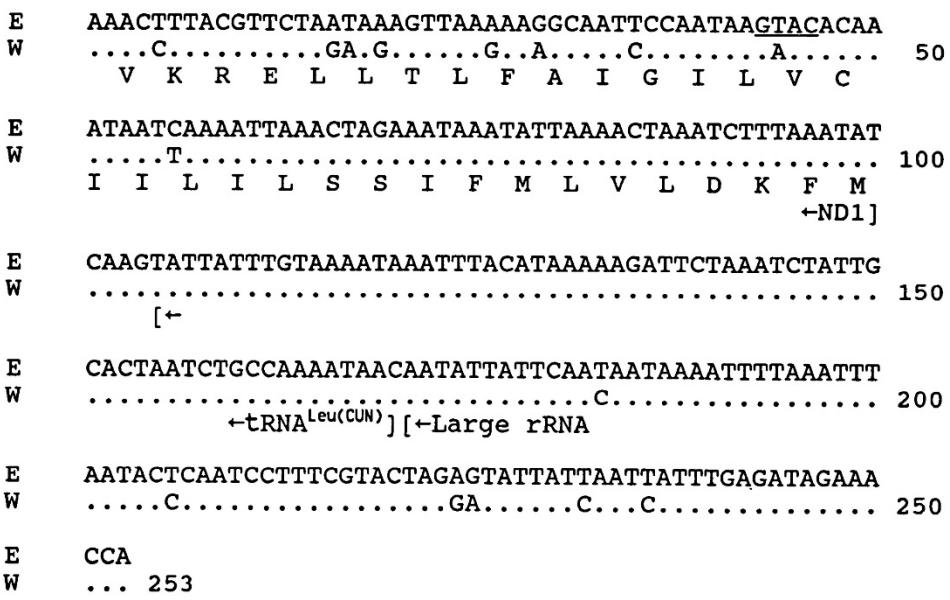

Figure 3. Sequence of Nl/LR amplicon obtained with primers Nl-J-12585 and LR-N-12854 for eastern and western strains of alfalfa weevil. Dots indicate identity. A potentially diagnostic *Rsa*I restriction site is underlined. Egyptian weevil sequence was identical to eastern and is not shown. Sequence corresponds to nucleotides 12586–12853 in *D. yakuba*.

Weevil strains differed by 9 silent substitutions in ND1, of which 7 were transitions. The t-RNA$^{Leu(CUN)}$ gene was identical in all weevil strains. Weevil strains differed by 6 transitions in the sequenced 3′ end of the large ribosomal gene.

Table 1 summarizes sequence differences between eastern and western haplotypes. Overall, the western haplotype was 5% divergent from the eastern and Egyptian haplotypes. Of the 52 nucleotide differences between weevil strains, 44 occurred in protein genes, resulting in 6% divergence between weevil strains in protein genes. Only 4 amino acid replacements, all in the ND1 gene, were observed among weevil strains.

Table 1. Mitochondrial sequence differences between eastern and western strains of alfalfa weevil

Gene	Base pairs	Ti/Tv[a]
CO-I	157	6/0
CO-II	256	10/2
Cytob	111	5/2
BD1	202	16/3
LrRNA	83	6/0
$tRNA^{leu(UUR)}$	65	1/0
$tRNA^{leu(CUN)}$	66	0/0
$tRNA^{ser(UCN)}$	68	0/1
Intergenic	23	0/0
Totals	1,031	44/8

a. Ti, transitions; Tv, transversions

Restriction Sites

Each of the 3 sequenced amplicons revealed potentially diagnostic restriction sites for differentiating eastern and Egyptian haplotypes from the western haplotype. The only nucleotide difference which might distinguish eastern and Egyptian weevils did not result in a restriction site.

The C1/C2 amplicon (618 bp including primers) had two *Alu*I sites in the eastern haplotype (284, 203, 131 bp fragments) and 1 site in the western haplotype (487, 131 bp fragments) (Fig. 4).

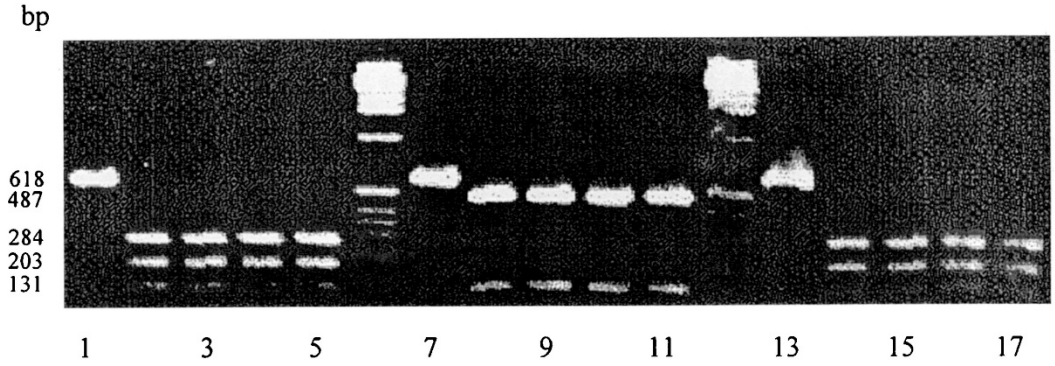

Figure 4. C1/C2 amplicon digested with *Alu*I. Eastern weevils from Maryland (lanes 2–5) and Egyptian from Arizona (lanes 14–17) have 2 sites resulting in 3 fragments (284, 203, 131 bp), western weevils from Utah (lanes 8–11) a single site resulting in 2 fragments (487, 131 bp). Lanes 1, 7, and 13 are uncut amplicons; lanes 6 and 12 are molecular-size standards.

The CB/N1 amplicon (349 bp including primers) produced 3 fragments (143, 123, 83 bp) in eastern weevils when cut with *Rsa*I and 2 fragments (206, 143 bp) in western weevils

(Fig. 5). The sequence difference detected between eastern and Egyptian weevils in this amplicon did not result in a potentially diagnostic restriction site.

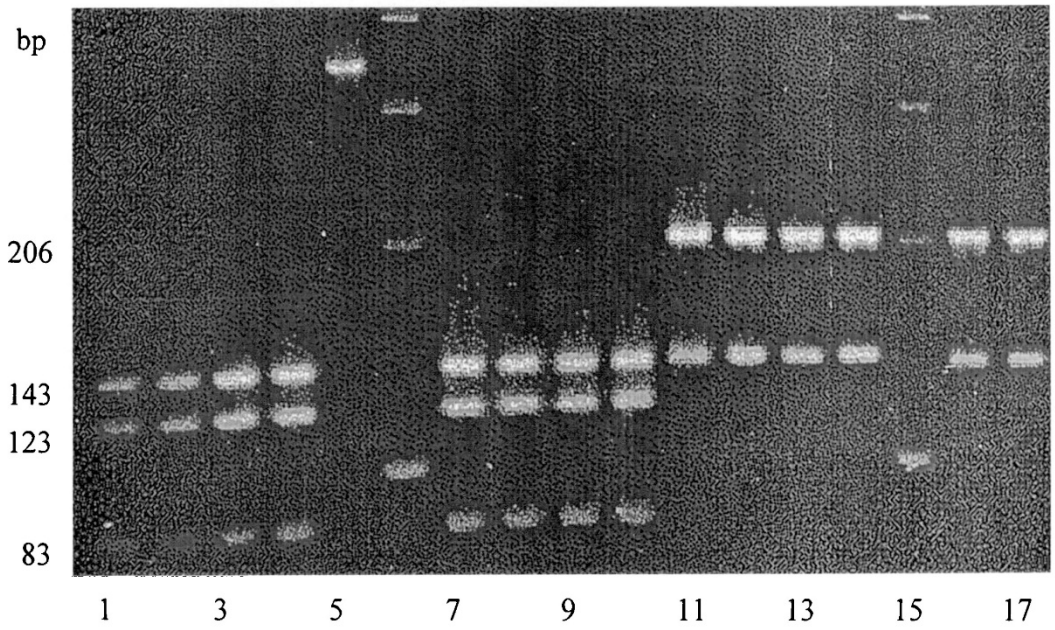

Figure 5. CB/N1 amplicon digested with *Rsa*I. Lanes 1 and 2 are Egyptian weevils from Arizona, lanes 3 and 4 eastern weevils from Maryland, lane 5 is uncut, lanes 6 and 15 are molecular-size standards, lanes 7–10 are weevils from Lincoln, lanes 11–14 are weevils from Scottsbluff, lanes 16 and 17 are western weevils from Utah. Eastern and Egyptian weevils have 2 sites yielding 3 fragments (143, 123, 83 bp), western weevils have 1 site yielding two fragments (206, 143 bp).

The LR/N1 amplicon (306 bp) similarly had 2 *Rsa*I sites in the eastern haplotype, giving 3 fragments (174, 72, 60 bp) but 1 site in western resulting in 2 fragments (246, 60 bp) (Fig. 6).

No exceptions were found in the digestion pattern of any of the amplicons. In all cases, eastern and Egyptian weevils gave identical results but differed from western weevils.

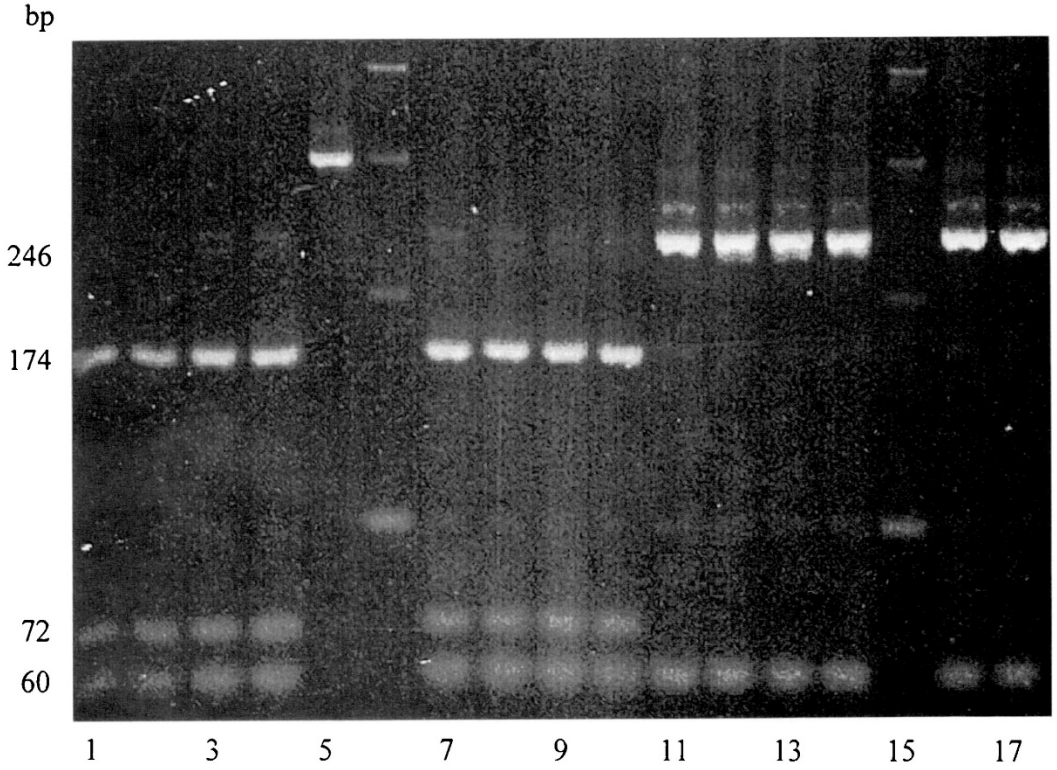

Figure 6. N1/LR amplicon digested with *Rsa*I using same weevils shown in Figure 5. Eastern and Egyptian weevils have 2 sites yielding three fragments (175, 72, 60 bp), western weevils have a single site yielding 2 fragments (246, 60 bp).

LR/N1 (Fig. 4) and CB/N1 (Fig. 5) amplicons from 150 weevils collected in Nebraska were digested with *Rsa*I. All 150 weevils were consistent for both diagnostic *Rsa*I restriction sites. Thirty-eight weevils (19 of each haplotype) from fields in which both eastern and western haplotypes were present sympatrically (as determined with restriction digests of the Nl/LR and CB/N1 amplicons) were scored for the diagnostic *Alu*I site in C1/C2 (Fig. 4). Results were consistent with those of the 2 other amplicons; i.e., each individual was assigned to the same strain by all 3 restriction digests. In Nebraska, the western haplotype was fixed at Scottsbluff and the eastern haplotype fixed at Lincoln. Both haplotypes were present sympatrically (same fields) in a broad area in central Nebraska (Table 2).

Table 2. Distribution of eastern and western strains of alfalfa weevil in Nebraska based on mitochondrial haplotypes found by cutting amplified DNA with 2 diagnostic restriction enzymes

	Strain	
County[a]	Eastern	Western
Lancaster	14	0
Seward	12	2
Cuming	4	1
Dixon	8	4
Hamilton	9	2
Buffalo	5	0
Franklin	27	1
Harlan	8	0
Furnas	15	5
Red Willow	14	4
Scotts Bluff	0	15
Totals	116	34

a. Order of locations is from east to west.

The sequence identity between putative eastern and Egyptian strains of the alfalfa weevil supports the opinion of Hsiao and Stutz (1985) that the 2 strains represent a single species. Blickenstaff (1965) suggested that eastern and western weevils deserve at least subspecies status. The amount of sequence variation (5%) which we found between western and eastern (or Egyptian) strains suggests a long divergence which could well be consistent with separate species status. Our estimate of sequence divergence is greater than that reported by Hsiao (1996) because of the fact that we sequenced more rapidly evolving regions of the mitochondrial genome. Valid species often differ by < 5%. Based on less information, within-species divergence is usually < 3%, but Brown et al. (1994) found 5.7% divergence between 2 individuals of *Greya obscura* Davis, Pellmyr & Thompson (Lepidoptera: Prodoxidae) taken from 2 populations. Although mitochondrial DNA can answer the question of maternal ancestry of individual weevils, alone it is not adequate for determining if hybridization is occurring in the field. The distribution of the 2 strains seems to have changed little since 1977 when, based on hybridization studies, Klostermeyer and Manglitz (1979) found nonhybrid eastern and western weevils sympatrically.

Our results show that although a relatively large genetic distance exists between alfalfa weevil strains, little variation is present in the mitochondrial genome within strains. This observation is consistent with a bottleneck effect associated with the recent introduction of small populations. The alfalfa weevil offers an opportunity to study genetic variability in an introduced species, presumably with small initial population sizes, followed by subsequent possibilities of genetic recombinations.

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