1996

Molecular Diagnostics of Three *Diabrotica* (Coleoptera: Chrysomelidae) Pest Species

Allen L. Szalanski
University of Nebraska-Lincoln

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

JOURNAL OF THE KANSAS ENTOMOLOGICAL SOCIETY
69(3), 1996, pp. 260–266

Molecular Diagnostics of Three *Diabrotica* (Coleoptera: Chrysomelidae) Pest Species

ALLEN L. SZALANSKI AND THOMAS O. POWERS
Department of Plant Pathology, University of Nebraska–Lincoln,
Lincoln, Nebraska 68583

ABSTRACT: A 257 bp region of the mitochondrial ND4 gene was analyzed for genetic variation in three species of corn rootworm, southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber, SCR), northern corn rootworm (*D. barberi* Smith and Lawrence, NCR), and western corn rootworm (*D. virgifera virgifera* LeConte, WCR). Nucleotide sequencing revealed 26 polymorphic sites. Genetic distances averaged 8% for all pair-wise comparisons among the three species. Restriction maps were constructed from sequence data and compared to potential species specific restriction sites. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) revealed three restriction enzymes (*Alu* I, *Apo* I and *Sau* 3A) which produced diagnostic patterns for both adults and larvae. Only NCR showed intraspecific polymorphism.

Corn rootworms are a complex of species in the genus *Diabrotica* and are the most serious pests of corn in midwestern United States (Levine and Oloumi-Sadeghi, 1991). Three important species are the southern corn rootworm (SCR), *Diabrotica undecimpunctata howardi* Barber; the northern corn rootworm (NCR), *D. barberi* Smith and Lawrence; and the western corn rootworm (WCR), *D. virgifera virgifera* LeConte. Adults of these species are readily differentiated by morphological characteristics. However, identification of the larvae can be difficult (Krysan, 1986), particularly if they are damaged. The anatomical similarity of larvae has stimulated the development of biochemical methods for rootworm identification. Allozymes have been evaluated in several studies of *Diabrotica,* including assessments of intraspecific (Krafsur, 1995; Krafsur et al., 1993; McDonald et al., 1985), and interspecific (Krysan et al., 1989; Piedrahita et al., 1985) variation. Allele frequencies and unique alleles differentiate rootworm species. Unfortunately, allozyme analysis requires samples to be frozen (−80°C) to prevent degradation, and can be affected by variation in enzyme expression during an insect life cycle. Also many species-specific alleles are present at low frequencies, requiring the sampling of many individuals and making individual identifications impossible. Cuticular hydrocarbons have also been employed to distinguish species but, profiles vary with stage, sex, and age of the insect (Golden et al., 1992).

Molecular genetic techniques can overcome some of the problems associated with the biochemical techniques. Insect samples can be collected and stored in alcohol (Sperling et al., 1994) and DNA characteristics are independent of life-stage or environment. DNA extracted from small amounts of tissue can be used for molecular analysis using polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLPs) (Crozier, 1993). Therefore tissue such as genitalia can be saved as morphological vouchers. PCR-RFLP has been successfully used for molecular diagnostics of gypsy moth (Pfeifer, 1995), blow flies (Sperling et al., 1994), and screwworm (Taylor et al., 1996).

The purpose of this study was to access a variable mtDNA molecular marker

Accepted for publication 8 May 1996.

Table 1. Source and stage of development of specimens analyzed.

Species	Location	Date collected	Origin	Stage	Number sampled[b]
D. barberi (NCR)	Talmage, NE	07/21/94	Field	Adult	1
	Lincoln, NE	07/11/94	Field	Adult	6
	Brookings, SD[a]	07/15/95	Field	Adult	8
D. howardi (SCR)	Lincoln, NE[a]	07/08/94	Field	Adult	6
	Lincoln, NE	07/02/94	Colony	Larvae	2
D. virgifera (WCR)	Lincoln, NE	07/08/94	Field	Adult	6
	Hickman, NE	07/14/94	Field	Adult	3
	Mead, NE	07/02/94	Colony	Larvae	2
	Brookings, SD[a]	07/15/95	Field	Adult	8

[a] One individual from this population was sequenced, NCR-A was the sequence haplotype or NCR.
[b] Used for PCR-RFLP.

as a source of diagnostic polymorphism for three species of rootworm. This research also provides data for molecular systematics of *Diabrotica.*

Materials and Methods

Collection of Specimens

Sample sites for adult rootworms were in southeastern Nebraska and eastern South Dakota (Table 1). Samples were transported to the laboratory alive and stored at −80°C until analysis. Larvae of SCR and WCR from a laboratory colony (originated from samples collected from southeastern Nebraska) maintained at the University of Nebraska–Lincoln Entomology Department were also analyzed (Table 1).

DNA Isolation

DNA was extracted from individual adult thoraces or from entire larvae using a phenol/choloroform extraction technique similar to that of Taylor et al. (1996). DNA pellets were resuspended in 50 μl pH 7.5 Tris:EDTA (TE) and stored at −20°C until use.

PCR Amplification

The mtDNA primers, N4-J-8502: 5′-GTAGGAGGAGCTGCTATATTAG-3′ and N4-N-8718: 5′-GCTTATTCATCGGTTGCTCA-3′ amplify a portion of the ND4 gene (Simon et al. 1994). Primers were synthesized by the University of Nebraska–Lincoln primer synthesis lab (Lincoln, NE). PCR reactions were conducted in 500 μl tubes using GeneE thermocycler (Techne Ltd., U.K.). Reaction mixture consisting of 1.0 μl of target DNA (from the 50 μl isolation), 5.0 μl of reaction buffer, 4.0 μl of dNTP mix (10 mM each of dATP, dTTP, dCTP and dGTP), 1.0 μl (20 mM) of each primer, 2.5 units of *Taq* polymerase and deionized water to a total volume of 50.0 μl. Reaction buffer, dNTP and *Taq* were obtained from a GeneAmp PCR reagent kit (Perkin Elmer Cetus, Norwalk, CT). The PCR amplification profile consisted of 35 cycles of 94°C for 45 sec, 50°C for one min, and 72°C for 1.5 min). Product was stored at 4°C.

```
Species          10         20         30         40         50
NCR       GTAGGAGGAG CTGCTATATT AGAAGATAAT AATAAAAATA TTCATAATCT
SCR       GTAGGAGGAG CTGCTATATT AGATGATAAT AATAAAAATA TTCATAATCT
WCR       GTAGGAGGAG CTGCTATATT AGAAGATAAT AATAAAAATA TTCATAATCT
                  Alu I
                    60         70         80         90        100
NCR       TAAATTGGGT ATAATATTCA ATAAGCCCTT ATTTAAATAA ATTCTACGGC
SCR       TAAATTAGGT ATAATATTTA ATAAGCCCTT ATTTAAATAA ATTCTTCGAC
WCR       TAAATTAGGT ATAATATTTA ATAAACCCTT ATTTAAATAA ATTCTTCGAC
                                          Dra I    Apo I
                   110        120        130        140        150
NCR       TATGAACACG CTCATAAAAA ATATTTGCTA AACAAAATAA ACCTGAAGAA
SCR       TATGAATACG TTCATAAAAA ATATTGGCCA AACAAAATAA ACCTGAAGAA
WCR       TATGAATACG TTCATAAAAA ATATTTGCTA AACAAAATAA ACCCGAAGAA

                   160        170        180        190        200
NCR       CATAACCCAT GAGCTAATAT CATAAATAAA GCTCCTCAAA ATCCTCATAT
SCR       CATAAACCAT GAGCTAATAT TATAAATAAG GATCCTCAAA ACCCTCATAT
WCR       CATAAACCAT GAGCTAATAT TATAAATAAA GCACCTATAA ATCCTCATAT
                     Alu I                Alu I Sau 3A
                   210        220        230        240        250
NCR       ATTTATCGTT AAAATTCCTC CTAATACTAA TCTTATATGA GCAACCGATG
SCR       ATTTAAGGTT AAAATACCAC CTAAAACCAA TCTTATATGA GCAACCGATG
WCR       ATTTATAGTT AAAATACCCC CTAATACTAA CCTTATATGA GCAACCGATG
                     Apo I
                   257
NCR       AATAAGC
SCR       AATAAGC
WCR       AATAAGG
```

Genbank accession numbers:

NCR: U63088; SCR: U63087; WCR: U63089

Fig. 1. Alignment of corn rootworm mitochondrial ND4 sequences. Restriction enzyme recognition sites (*Alu* I, *Apo* I and *Sau* 3A) are highlighted, and primer sequences are underlined. Genbank accession numbers are listed for each sequence.

DNA Sequencing

PCR product was sequenced from one individual for each rootworm species (Table 1). Amplified DNA was purified using *Geneclean II* (Bio 101, Inc. Vista, CA) following manufacturer directions and resuspended in 30 μl of TE (pH 7.5). Amplified DNA was blunt ended using New England Biolabs (Beverly, MA) reagents and ligated using Stratagene (La Jolla, CA) reagents into pBluescript sk+ plasmid (Stratagene, La Jolla, CA) following Sambrook et al. (1989). Plasmid transformation and screening of clones were conducted following Sambrook et al. (1989). Positive colonies were replated (Sambrook et al. 1989) and verified by

PCR. PCR reaction was performed as before but for only 20 cycles. Five μl of PCR product was run on an agarose gel to determine if the insert was present. Single stranded product was obtained from positive clones and sequenced using a LI-COR Model 4000 DNA Sequencer (LI-COR Inc., Lincoln, NE) by the University of Nebraska-Lincoln DNA Sequencing Lab (Lincoln, NE). Two primers, T3 promotor and T7 promotor (Gibco BRL, Gaitherburg, MD), were used to sequence in both directions. The Genbank Accession Numbers for the ND4 mtDNA sequence for each species are in Fig. 1.

Sequence Alignments

Sequences were aligned using GCG (Genetics Computer Group) PILEUP program (with a gapweight of 5.0 and a gaplengthweight of 0.3). Pairwise absolute distances between species were calculated using PAUP 3.11 (Swofford 1991).

PCR-RFLP

Restriction sites were predicted from sequence data using the program MAP in GCG. Sample DNA was amplified as previously described. Predicted diagnostic markers were checked with restriction enzymes from New England Biolabs (Beverly, MA). Digestion and gel electrophoresis of fragments were conducted per Taylor et al. (1996).

Results and Discussion

The mtDNA fragment was readily amplified using adults and larvae in all of our samples. The ND4 amplicon sequence was 215 bp in length (excluding primers) for all samples (Figure 1). Twenty-six nucleotide sites were polymorphic among the three taxa. Pairwise absolute distances (Swofford 1991) between rootworm taxa for ND4 amplicon were: NCR-SCR, 0.093; NCR-WCR, 0.079; and SCR-WCR, 0.079.

Digestion of the ND4 amplicon with five commercially available restriction enzymes produced patterns that were interpretable on agarose gels (Table 2), although only three of five tested enzymes proved useful. *Ssp* I consistently gave partial digests, which obscured interpretation of RFLP patterns. *Mse* I produced too many small fragments for accurate visualization of polymorphisms using agarose gel electrophoresis. Three restriction enzymes, *Alu* I, *Apo* I, and *Sau* 3A, were useful for distinguishing the three species (Table 2). SCR and WCR had two *Alu* I sites which were fixed in all samples (Figure 2). NCR had three *Alu* I sites and two unique *Alu* I patterns (NCR-A, NCR-B), both of which were present in Nebraska and South Dakota populations (Figure 2). Nine individuals had pattern A and 6 had pattern B. *Apo* I recognizes a common site at nucleotide 89 for all rootworms tested, but an A → T transversion created a second *Apo* I site in NCR (Table 2). *Sau* 3A did not digest the NCR-A and WCR amplicon, but a C → A transversion created a site at nucleotide 180 in SCR and NCR-B haplotype (Figure 3). NCR was the only species with intraspecific polymorphism. Larva and adult patterns were identical for SCR and WCR.

It appears that sufficient divergence exists in this mtDNA region for a large-scale assessment of diagnostic polymorphism. Surveys will be required to extend these results to a broader geographic region. Because larvae of several corn rootworm species often occur together on the same host, and may be damaged during extraction from the host, PCR techniques provide a valuable tool for their iden-

Table 2. Rootworm restriction fragment sizes from 257 bp mtDNA amplicon.

Restriction enzyme	Species	Position of recognition site	Predicted size of resultant fragment (bp)
Alu I	NCR-A	10, 163, 181	10, 153, 18, 76
	NCR-B	163	163, 94
	SCR	10, 163	10, 153, 94
	WCR	10, 163	10, 153, 94
Apo I	NCR	89, 212	89, 123, 45
	SCR	89	89, 168
	WCR	89	89, 168
Sau 3A	NCR-A		257
	NCR-B	180	180, 77
	SCR	180	180, 77
	WCR		257
Mse I	NCR	50, 83, 209	50, 33, 126, 48
	SCR	50, 68, 83, 203, 209	50, 18, 15, 120, 6, 48
	WCR	50, 68, 83, 209	50, 18, 15, 126, 48
Ssp I	NCR	39, 65, 122	39, 26, 57, 135
	SCR	39, 65, 122, 168	39, 26, 57, 46, 89
	WCR	39, 65, 122, 168	39, 26, 57, 46, 89

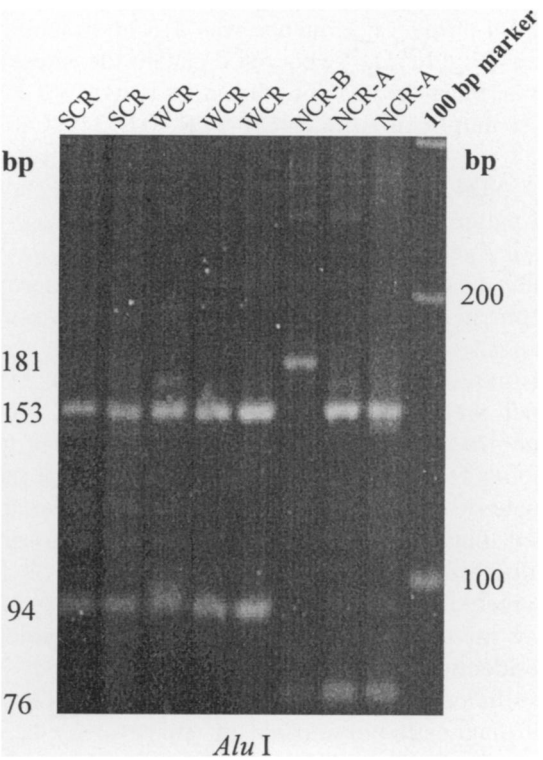

Fig. 2. *Alu* I restriction enzyme digests of amplicon ND4, a 257 bp region of the mitochondrial ND4 gene. SCR, *Diabrotica undecimpunctata howardi*; WCR, *D. virgifera virgifera*; NCR, *D. barberi*.

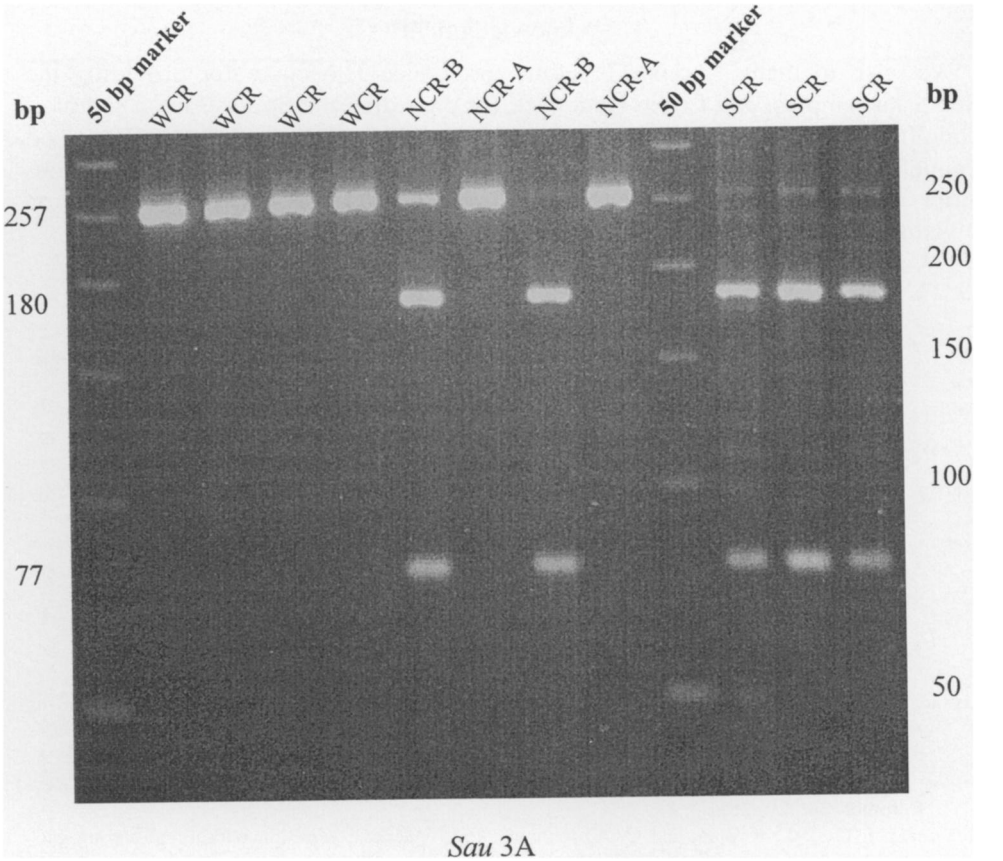

Fig. 3. *Sau* 3A restriction enzyme digests of amplicon ND4, a 257 bp region of the mitochondrial ND4 gene. SCR, *Diabrotica undecimpunctata howardi*; WCR, *D. virgifera virgifera*; NCR, *D. barberi.*

tification and study. Based on sequence data, PCR-RFLP can provide an economical and rapid identification of multiple specimens. Analysis by PCR-RFLP can be completed in 12–24 hours using expendables costing less than $2.50 per specimen (Taylor et al., 1996).

In addition to distinguishing between species, mtDNA variation may provide additional information on the phylogenetic relationships within *Diabrotica.* Based on allozyme variation, NCR and WCR are members of a common clade (virgifera) and SCR belongs to a separate clade (fucata) (Krysan et al., 1989). Rootworm relationships cannot be determined based only on the limited nucleotide data presented here. Yet there is enough sequence divergence in the mitochondrial region to suggest that an expanded taxonomic survey would be phylogenetically informative. Future work involving other *Diabrotica* species, additional molecular markers and spatially separated populations will provide greater insight into phylogenetic relationships and population genetics of these species.

Acknowledgment

We wish to thank Thomas E. Hunt and Lance J. Meinke for providing the Nebraska samples, and Leslie Hammack for providing the South Dakota samples. Timothy Harris provided assistance with the gel electrophoresis. Leslie Lane and Kenneth Pruess provided helpful suggestions and critical reviews of the manuscript. This is Journal Paper No. 11484 of the Nebraska Agricultural Research Division.

Literature Cited

Crozier, R. H. 1993. Molecular methods for insect phylogenetics. *In* J. Oakeshott and M. Whitten (eds.), Molecular Approaches to Fundamental and Applied Entomology, pp. 164–221. Springer-Verlag, New York.

Golden, K. L., L. J. Meinke, and D. W. Stanley-Samuelson. 1992. Cuticular hydrocarbon discrimination of *Diabrotica* (Coleoptera: Chrysomelidae) sibling species. Ann. Entomol. Soc. Am. 85: 561–570.

Krafsur, E. S. 1995. Gene flow between univoltine and semivoltine northern corn rootworm (Coleoptera: Chrysomelidae) populations. Ann. Entomol. Soc. Am. 88:699–704.

Krafsur, E. S., P. Nariboli, and J. J. Tollefson. 1993. Gene diversity in natural *Diabrotica barberi* Smith and Lawrence populations (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Am. 86: 490–496.

Krysan, J. L. 1986. Introduction: biology, distribution, and identification of pest *Diabrotica*. *In* J. L. Krysan and T. A. Miller (eds.), Methods for the Study of Pest *Diabrotica*, pp. 1–23. Springer-Verlag, New York.

Krysan, J. L., I. C. McDonald, and J. H. Tumlinson. 1989. Phenogram based on allozymes and its relationship to classical biosystematics and pheromone structure among eleven diabroticites (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Am. 82:574–581.

Levine, E., and H. Oloumi-Sadeghi. 1991. Management of diabroticite rootworms in corn. Ann. Rev. Entomol. 36:229–255.

McDonald, I. C., J. L. Krysan, and O. A. Johnson. 1985. Genetic variation within and among geographic populations of *Diabrotica barberi* (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Am. 78:271–278.

Pfeifer, T. A., L. M. Humble, M. Ring, and T. A. Grigliatti. 1995. Characterization of gypsy moth populations and related species using a nuclear DNA marker. Can. Entomol. 127:49–58.

Piedrahita, O., C. R. Ellis, and J. P. Bogart. 1985. Electrophoretic identification of the larvae of *Diabrotica barberi* and *D. virgifera virgifera* (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Am. 78:537–540.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd Ed., 3 Volumes. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87:651–701.

Sperling, F. A. H., G. S. Anderson, and D. A. Hickey. 1994. A DNA-based approach to the identification of insect species used for postmortem interval estimation. J. Forensic Sci. 39:418–427.

Swofford, D. 1991. "PAUP": Phylogenetic Analysis Using Parsimony, Version 3.0s. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Taylor, D. B., A. L. Szalanski and R. D. Peterson, II. 1996. Identification of screwworm species (Diptera: Calliphoridae) by polymerase chain reaction—restriction fragment length polymorphism. Med. Vet. Entomol. 10:63–70.