2001

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Thomas L. Clark
University of Nebraska-Lincoln, clarkth@missouri.edu

Lance J. Meinke
University of Nebraska-Lincoln, lmeinke1@unl.edu

John E. Foster
University of Nebraska-Lincoln, john.foster@unl.edu

Bulletin of Entomological Research (2001) **91**, 419–427 DOI: 10.1079/BER2001130

PCR–RFLP of the mitochondrial cytochrome oxidase (subunit I) gene provides diagnostic markers for selected *Diabrotica* species (Coleoptera: Chrysomelidae)

T.L. Clark*, L.J. Meinke and J.E. Foster

Insect Genetics Laboratory, Department of Entomology, University of Nebraska-Lincoln, Lincoln, NE 68583-0816, USA

Abstract

Adult and larval identification of *Diabrotica* can be difficult. Some adult identifications require considerable taxonomic experience while larvae of many *Diabrotica* species are morphologically indistinguishable. This study was conducted to determine whether 12 pest and non-pest *Diabrotica* species could be separated using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). A 1308 bp portion of the mitochondrial cytochrome oxidase subunit I gene (COI) was amplified using PCR and digested using several restriction endonucleases. Double digests of COI amplicons with *Alu*I and *Msp*I resolved on polyacrylamide gels revealed several diagnostic inter- and intraspecific polymorphisms. A key to the 12 species was constructed using the PCR–RFLP patterns.

Introduction

The genus *Diabrotica* (Coleoptera: Chrysomelidae) includes several important pest species, including the western corn rootworm, *D. virgifera virgifera* LeConte and northern corn rootworm, *D. barberi* Smith & Lawrence (Krysan, 1986). The genus is generally divided into three species groups: *virgifera*, *fucata* and *signifera* (Smith & Lawrence, 1967) with the *virgifera* and *fucata* species groups containing important pest species (Krysan, 1986). Species within the *virgifera* group are uni- or semivoltine (Krysan *et al*., 1986), with diapause occurring in the egg stage during either the winter months in temperate zones or the dry season in tropical regions (Krysan, 1982). This trait is hypothesized to have evolved in an ancestral species as a response to alternating wet and dry seasons, because larvae fed on roots of certain grasses that were available during the wet season only (Branson & Krysan, 1981). Distribution of species in the *virgifera* group is limited by distribution of obligatory larval host plants primarily in the family Poaceae (Smith, 1966; Branson & Ortman, 1970; Branson & Krysan, 1981). In contrast, species in the *fucata* group are multivoltine and overwinter as adults (Marsh, 1910; Arant, 1929) with adults and larvae being polyphagous on several plant families (Branson & Krysan, 1981). A striking example of the voltinism differences between the two species groups is evident where the univoltine *D. v. virgifera–D. v. zeae* Krysan & Smith, subspecies complex occurs in the same geographic region where *fucata* species group taxa may have up to six generations per year (Krysan, 1986)

There are at least 15 species and subspecies of *Diabrotica* that are reported pests of 61 different crops (Krysan, 1986). Primarily, maize, cucurbits, sweet potatoes and legumes are the hosts of *Diabrotica*. Damage to these crops can be economically devastating. For example, three rootworm species, *D. v. virgifera*, the western corn rootworm; *D. barberi*,

* Address for correspondence: Department of Entomology, 1–87 Agriculture Building, University of Missouri, Columbia, MO 65211, USA
Fax: (573) 882 1469
E-mail: clarkth@missouri.edu

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). Up to an additional $100 million is lost to *Diabrotica* sp. 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).

Diabrotica are historically neotropical with the greatest diversity of species occurring in tropical areas (Krysan & Smith, 1987). While many pest species exist in this genus there are several non-pest species of which little is known about their biology. As a result, little information exists as to which plant species are utilized by either pest or non-pest *Diabrotica* beyond agricultural crops. The deficiency of biological information on *Diabrotica* can be partially attributed to a lack of identification tools for all life stages. Most known species can be identified as adults using available keys (Krysan, 1986; Krysan & Smith, 1987). However, adults of some species require extensive morphological evaluation for proper identification. For example, questionable males of the Mexican corn rootworm, *D. v. zeae* Krysan & Smith, are distinguished from *D. porracea* Harold males by width to length ratio of the aedeagus as well as being generally larger in size (Krysan & Smith, 1987). Likewise, many known pest species can be differentiated by external sculpturing of the egg chorion (Atyeo *et al.*, 1964; Rowley & Peters, 1972; Krysan, 1986).

Attempts to distinguish larvae using external characters have proven to be difficult (Krysan, 1986). Mendoza & Peters (1964) devised a key to differentiate mature *D. u. howardi*, *D. v. virgifera* and *D. barberi* larvae. No other larval key is currently available. Furthermore, Krysan (1986) observed that third instar larvae from the *fucata* and *virgifera* species groups can readily be separated based on various characters such as the presence (*fucata*)/absence (*virgifera*) of urogomphi on the anal plate. Despite the key, Krysan (1986) reported that some specimens of the *D. v. virgifera* and *D. barberi* maize pest complex of the north-central United States cannot be separated with certainty because distinguishing features vary in some larvae. Because these pests are sympatric (often occupying the same field with variable species ratios), Krysan's (1986) observation is important from a pest management perspective, as *D. v. virgifera* (unlike *D. barberi*) is notorious for its ability to develop high levels of resistance to some organochlorine, organophosphate and carbamate insecticides (Ball & Weekman, 1962; Meinke *et al*., 1998). Piedrahita *et al*. (1985) conducted a horizontal starch electrophoresis survey of 20 enzyme systems to discriminate between *D. v. virgifera* and *D. barberi* larvae (second and third instars). Morphologically, they observed that third instar *D. barberi* were misidentified as *D. v. virgifera* 52% of the time, while *D. v. virgifera* were misidentified as *D. barberi* 5.8% of the time. While Piedrahita *et al*. (1985) provided a useful tool for distinguishing between *D. v. virgifera* and *D. barberi*, their results were variable between instars and insects used in their assay lost enzymatic activity quickly upon death. This is a common constraint with allozyme studies as specimens must be maintained in frozen conditions to maintain enzyme activity (West *et al*., 1997)

Another diagnostic method used to identify insect species is PCR–RFLP (polymerase chain reaction–restriction fragment length polymorphism). This method has been successfully used to separate morphologically indistinguishable hepialid moths in the genus *Wiseana* (Lepidoptera: Hepialidae) (Brown *et al*., 1999), invasive tephritid fruit flies (Diptera: Tephritidae) for quarantine applications (Armstrong *et al*., 1997), and the morphologically cryptic malaria vector *Anopheles minimus* Theobold species complex (Diptera: Culicidae) (Van Bortel *et al*., 2000). West *et al*. (1997) used the technique to discriminate 13 *Aedes* species by developing a dichotomous key based upon the resulting fragment migration patterns. Szalanski & Powers (1996) previously attempted to distinguish between *D. v. virgifera*, *D. barberi* and *D. u. howardi* using PCR–RFLP of a 257 bp portion of the mitochondrial NADH dehydrogenase subunit 4 (ND4) gene. However, they were unable to distinguish successfully between the three species using single restriction enzymes. The purpose of this study was to differentiate 12 *Diabrotica* species using PCR–RFLP markers and to devise a diagnostic key based upon the resulting restriction digest fragment patterns.

Materials and methods

Insects and DNA extraction

Beetles were collected from several sites during the summers of 1997–1999 (table 1, fig. 1). They were identified morphologically using dichotomous keys (Krysan, 1986; Krysan & Smith, 1987) and then verified by J. Krysan (United States Department of Agriculture-Agricultural Research Service, retired). Identified specimens were either placed in 95% ethanol or frozen (−80°C). Representative voucher specimens have been deposited in the University of Nebraska State Museum, Lincoln, Nebraska, USA. Five larvae from each of the following species: *D. balteata*, *D. barberi*, *D. longicornis* (Say), and *D. speciosa* Germar, *D. u. howardi*, *D. u. undecimpunctata*, *D. v. virgifera* that were maintained in culture in our laboratory or sent from other laboratories were also examined to verify that PCR amplification would not be hindered by possible inhibitors present in larvae.

DNA was extracted from the thorax of individual beetles or whole larvae with the digestive tract and fatty tissue removed using a CTAB (hexadecyltrimethylammonium bromide) extraction method described by Black & Du Teau (1997).

PCR amplification

A portion of the mitochondrial cytochrome oxidase subunit 1 (COI) was amplified using universal primers from the COI (C1-J-1718 5′-GGATCACCTGATATAGCATTCCC-3′) and the tRNA leucine genes (TL2-N-3014 5′-TCCAATGCACTAATCTGCCATATTA-3′) (Simon *et al*., 1994). Gene amplification was done in 25 µl reaction volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM dNTP's, 0.8 µM of each primer, 1.25 units of Taq polymerase (Perkin Elmer, Branchburg, New Jersey, USA), 3.5 mM $MgCl_2$ and 3 µl of DNA template (diluted 1:10 from the original CTAB extraction). PCR reaction mixtures were then amplified using either a GeneAmp PCR system 2400 or 9600 (Perkin Elmer, Branchburg, New Jersey, USA) with the following temperature profile: a hold of 94°C for 2 min; 35

Table 1. Collection sites for *Diabrotica* specimens used for polymerase chain reaction–restriction fragment length polymorphism analysis.

Diabrotica species	n	Pest status	Collection site(s)*
virgifera species group			
D. barberi Smith & Lawrence	20	Yes	Iowa-Clinton County (**1**); Nebraska-Saunders County (**2**), Madison County (**3**); South Dakota-Brookings County (Bruce (**4**) and Volga Townships (**5**))
D. cristata (Harris)	20	No	Nebraska-Lancaster County (Arbor Road (**6**), 9-Mile Prairie (**7**), and Wulf Tallgrass Prairie (**8**))
D. lemniscata LeConte	20	No	New Mexico-Colfax County (**9**)
D. longicornis (Say)	20	Possible	Kansas-Scott County (**10**); Nebraska-Dundy County (**11**), Nuckolls County (**12**)
D. porracea Harold	10	Yes	Panama-Cordillera (**13**)
D. virgifera virgifera LeConte	20	Yes	Colorado-Bent County (**14**), Larimer County (**15**); Illinois-Champaign County (**16**); Indiana-St Joseph County (**17**); Kansas-Decatur County (**18**), Finney County (**19**), Wallace County (**20**); Nebraska-Dixon County (**21**), Dundy County (**11**), Franklin County (**22**), Nuckolls County (**12**)
D. v. zeae Krysan & Smith	20	Yes	Texas-Bell County (**23**)
D. viridula (Fabricius)	20	Yes	Brazil-Sete Lagoas (**24**); Panama-Cordillera (**13**)
fucata species group			
D. balteata LeConte	20	Yes	Florida-Indian River County (**25**)
D. speciosa Germar	20	Yes	Brazil-Sete Lagoas (**24**)
D. undecimpunctata howardi Barber	20	Yes	Colorado-Cheyenne County (**26**), Washington County (**27**); Nebraska-Lancaster County (**6**), Saunders County (**2**), Webster County (**28**); New Mexico-Colfax County (**9**)
D. u. undecimpunctata Mannerheim	20	Yes	California-Alameda County (**29**)

* Numbers in bold behind individual collection sites correspond to the location designation shown in fig. 1.

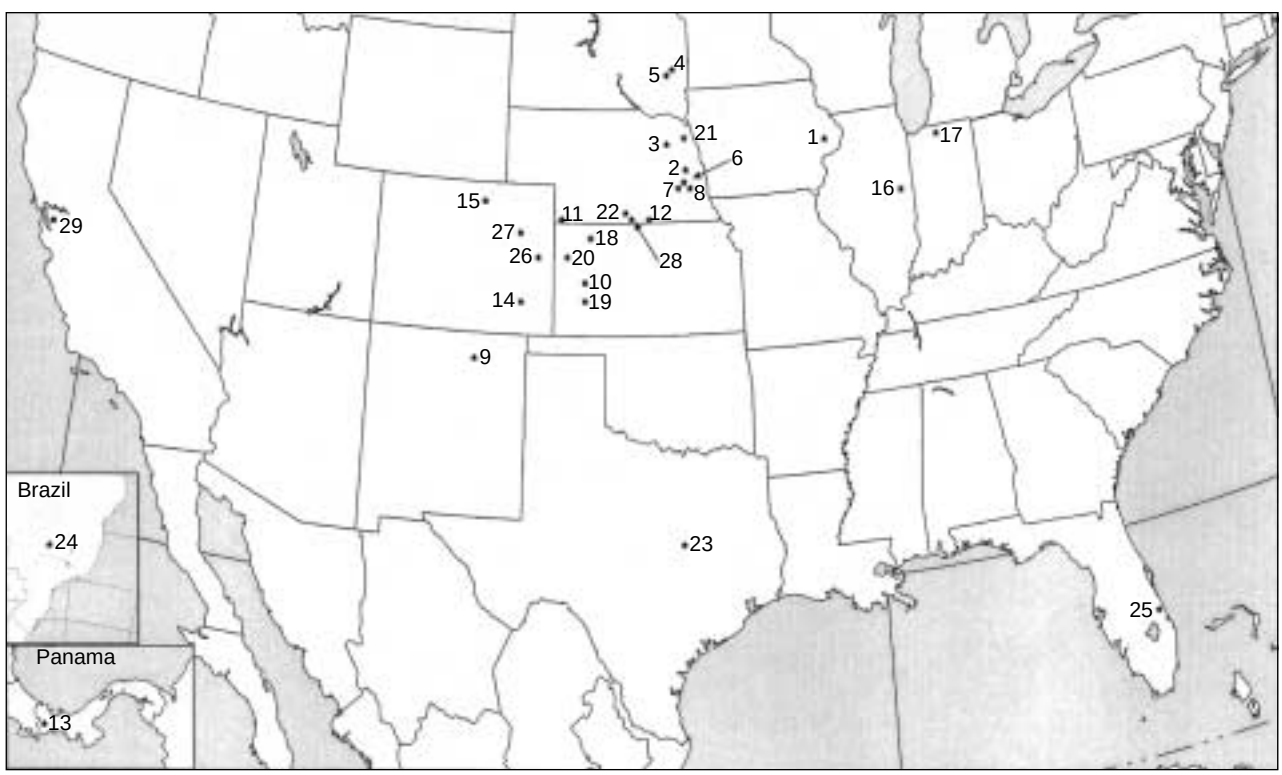

Fig. 1. Map of *Diabrotica* collection sites.

cycles of 94°C for 1 min, 52°C for 30 sec, 72°C for 1 min; and a final extension step for 8 min after the final cycle. All amplifications had a negative control containing no DNA template. PCR products (4 µl) were loaded onto 1.0% agarose TBE (0.089 M Tris, 0.089 M boric acid, 0.5 M EDTA (pH 8.0)) gels. After electrophoresis (75 V for 45 min), PCR amplicons were visualized over a UV transilluminator and scanned into the Advanced Quantifier gel documentation program (Genomic Solutions, Ann Arbor, Michigan, USA).

Restriction digests

PCR amplicons were screened using five 4-bp recognition restriction endonucleases (*Alu*I, *Dpn*II, *Mse*I, *Msp*I, *Rsa*I) and four 6-bp recognition restriction endonucleases (*Ase*I, *Dra*I, *Hpa*I, and *Xba*I) following the manufacturers protocol (New England BioLabs, Beverly, Massachusetts, USA). Two enzymes, *Alu*I and *Msp*I, used together in a double digest resulted in the best diagnostic fragment patterns for each *Diabrotica* species screened. Double digests were done in 15 µl reaction volumes consisting of 1.5 µl buffer 2 (50 mM NaCl, 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1mM DTT (New England BioLabs, Beverly, Massachusetts, USA)), 7.75 µl dd H_20, 4 µl PCR product, 1.0 µl *Alu*I (10 units) and 0.5 µl *Msp*I (10 units). The reaction mixture was then incubated at 37°C for 17–21 h in PCR reaction tubes on a GeneAmp PCR system 9600 (Perkin Elmer, Branchburg, New Jersey, USA). Digested PCR amplicons were fractionated (40 V for 14 h at 20°C) on 9% polyacrylamide (29:1 acrylamide:bis-acrylamide) (Sambrook *et al*., 1989) and silver stained (PlusOne DNA silver staining kit, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA).

Theoretical restriction digests

Complimentary to screening COI amplicons using restriction enzymes, restriction sites for all enzymes in the1308 bp COI region were examined using Webcutter 2.0 (Heiman, 1997) software for single specimens of each *Diabrotica* species included in this study that had been previously sequenced and deposited in GenBank as part of a phylogenetic analysis (Clark *et al*., 2001).

Diagnostic key

A dichotomous key was devised by comparing digested PCR amplicon fragment patterns to known DNA size markers (1Kb DNA ladder, Gibco BRL, Gaithersburg, Maryland, USA).

Results

The amplified portion of the COI gene for all species of *Diabrotica* was 1308 bp (fig. 2). Additionally, no differences in

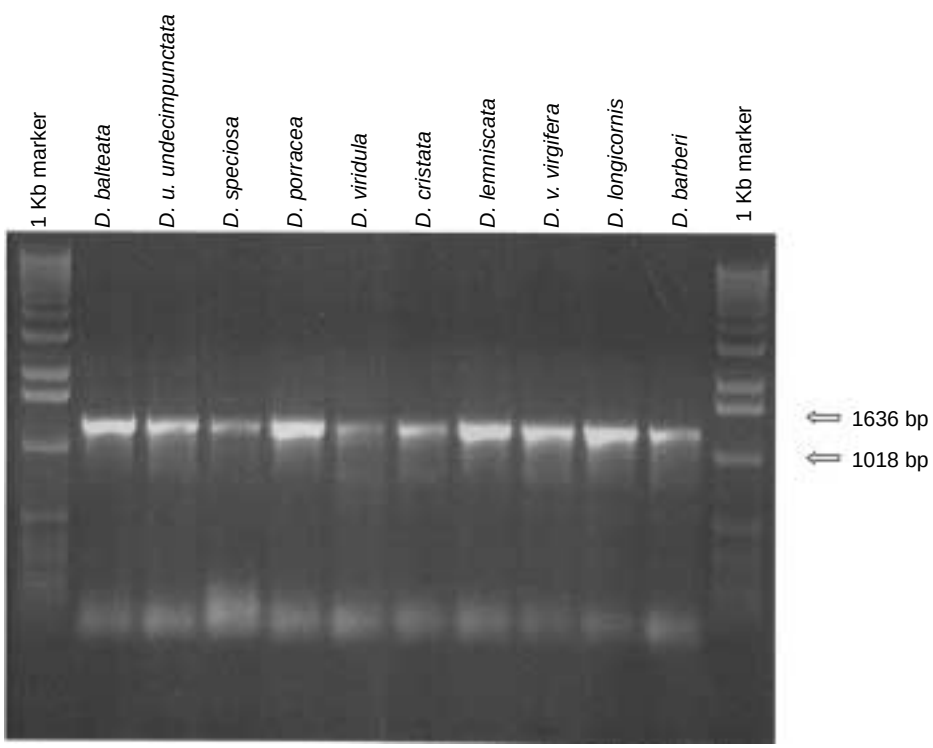

Fig. 2. Agarose gel of undigested 1308 bp of mitochondrial cytochrome oxidase subunit I amplicons of *Diabrotica* as amplified by polymerase chain reaction.

Table 2. Fragment sizes after amplification of 1308 bp of mitochondrial cytochrome oxidase subunit I for *Diabrotica* and double digestion with *Alu*I and *Msp*I as they appear on 9.0% polyacrylamide gels visualized with silver staining.

Diabrotica species	Polymorph	Fragment sizes
D. barberi	A	590, 360, 180, 97, 59
	B	630, 184, 137, 120, 97, 69, 59
	C	604, 335, 180, 59
	D	604, 184, 137, 97, 59
D. cristata	A	355, 180, 152, 140, 113, 101, 95, 59, 45, 40
	B	475, 180, 141, 125, 101, 59, 45, 40
	C	328, 180, 152, 140, 113, 101, 77, 59, 45, 40
D. lemniscata		448, 235, 175, 151, 120, 120, 107, 59
D. longicornis	A	604, 335, 180, 59
	B	604, 184, 137, 97, 59
	C	619, 360, 184, 97, 59
D. porracea		420, 206, 187, 178, 110, 102, 59, 52, 42
D. v. virgifera		313, 140, 131, 113, 101, 77, 66, 59, 41
D. v. zeae		313, 140, 131, 113, 101, 77, 66, 59, 41
D. viridula	A	676, 180, 145, 77, 60, 53
	B	676, 225, 180, 77, 60
D. balteata		455, 222, 179, 157, 98
D. speciosa	A	375, 220, 184, 126, 113, 104, 85, 50
	B	378, 180, 176, 126, 113, 104, 84, 50
	C	378, 180, 176, 148, 126, 113, 104, 98, 84, 50
D. u. howardi	A	518, 452, 183, 118, 98, 63, 58
	B	442, 224, 223, 183, 118, 98, 63, 58
D. u. undecimpunctata	A	518, 452, 162, 118, 98, 63, 58

Table 3. Generalized location of restriction sites and fragment sizes of 1308 bp of amplified mitochondrial cytochrome oxidase subunit I for selected *Diabrotica* species as indicated by Webcutter 2.0 software.

Diabrotica species	GenBank accession number	Position of restriction sites for *Alu*I	Position of restriction sites for *Msp*I	Predicted fragment sizes
D. barberi	AF278544	175, 220, 538, 604, 646, 712, 760	197	175, 22, 23, 318, 66, 42, 66, 48, 548
D. cristata	AF278545	175, 196, 298, 352, 538, 604, 646, 760, 814, 1162, 1184	337, 407	175, 21, 102, 39, 15, 55, 131, 66, 42, 114, 54, 348, 22, 124
D. lemniscata	AF278546	175, 298, 406, 604, 646, 712, 760, 1162	800	175, 123, 108, 198, 42, 66, 48, 40, 362, 146
D. longicornis	AF278547	same as *D. barberi*	same as *D. barberi*	same as *D. barberi*
D. porracea	AF278548	175, 298, 352, 538, 646, 712, 892, 1250	197	175, 22, 101, 54, 186, 108, 66, 180, 358, 58
D. v. virgifera	AF278549	175, 196, 298, 346, 352, 538, 604, 646, 712, 760, 892, 1162, 1240	407	175, 21, 102, 48, 6, 55, 131, 66, 42, 66, 48, 132, 270, 78, 68
D. v. zeae	AF278550	175, 196, 406, 538, 604, 646, 712, 760, 892, 1162, 1240	337	175, 21, 141, 69, 132, 66, 42, 66, 48, 132, 270, 78, 68
D. viridula	AF278551	175, 220, 298, 538, 604, 646, 712	347, 407	175, 45, 78, 49, 60, 131, 66, 42, 66, 596
D. balteata	AF278553	175, 538, 646, 712, 814, 862, 910	197, 347, 485	175, 22, 150, 138, 53, 108, 66, 102, 48, 48, 398
D. speciosa	AF278554	4, 175, 196, 298, 346, 604, 862, 892, 910, 1199	403, 485, 686	4, 171, 21, 102, 48, 57, 82, 119, 82, 176, 30, 18, 289, 109
D. u. howardi	AF278555	175, 814, 910	110, 347, 745	110, 65, 172, 398, 69, 96, 398
D. u. undecimpunctata	AF278556	175, 196, 814, 910	110, 347, 745	110, 65, 21, 151, 398, 69, 96, 398

amplifying the 1308 bp COI amplicon from either adult or larval DNA extractions were observed. After preliminary digests with several restriction enzymes and theoretical digests using Webcutter 2.0 software based on COI sequences in GenBank it was decided to digest the COI amplicon with the restriction enzymes *Alu*I and *Msp*I, simultaneously. Fragments resulting from the *Alu*I and *Msp*I double digest ranged in size from 676 to less than 40 bp with

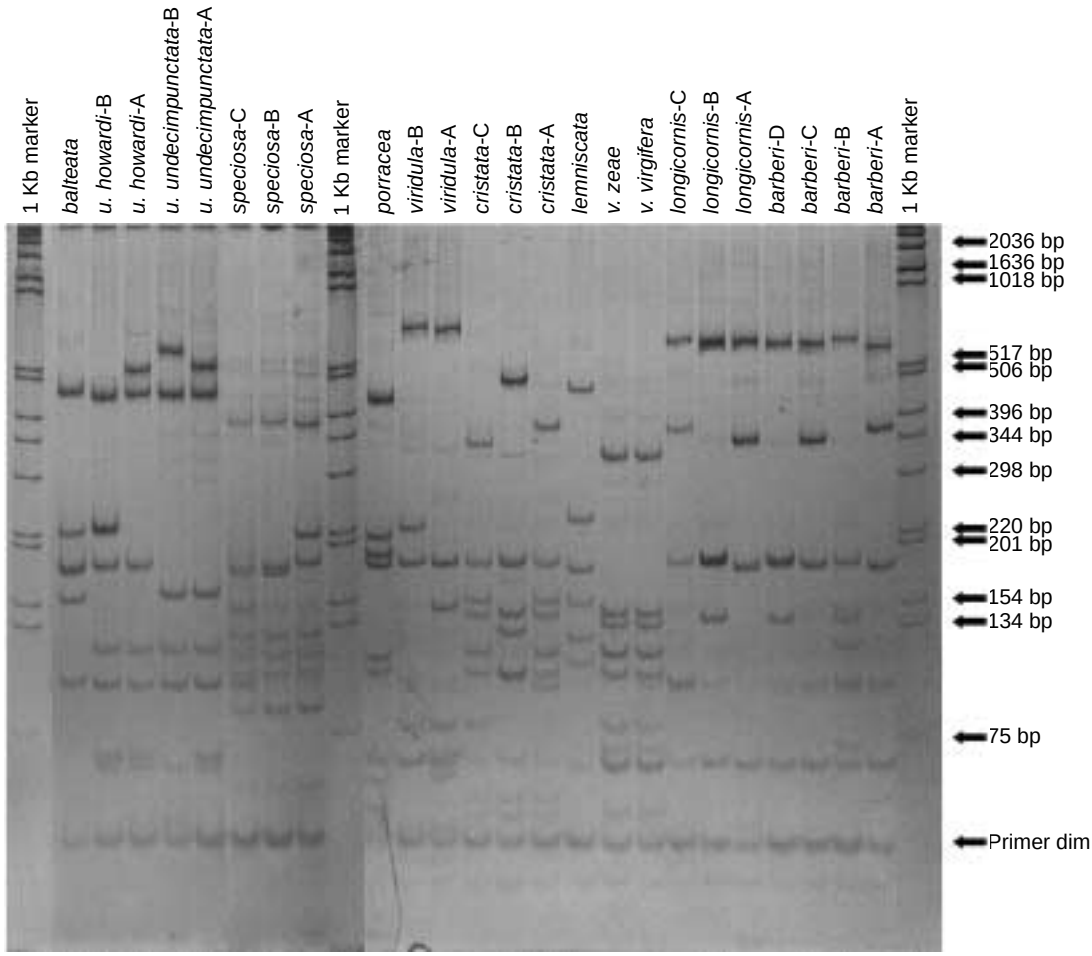

Fig. 3. Polyacrylamide gel of restriction fragment patterns for 12 *Diabrotica* species produced by *Msp*I and *Alu*I digestion of the of mitochondrial cytochrome oxidase subunit I amplicon visualized with silver staining.

≥4 fragments per species when fractionated on 9.0% polyacrylamide gels (table 2, fig. 3). Fragments less than 40 bp had poor resolution and were eliminated from further consideration. In other preliminary studies, agarose gels revealed diagnostic markers for many of the species. However, the resolving power of polyacrylamide was needed to differentiate fragments of smaller size. Because DNA migration on polyacrylamide gels is sensitive to base composition in addition to actual size (Sambrook *et al*., 1989), the total addition of fragments per lane in polyacrylamide gels was not equal to and generally greater than the 1308 bp of the undigested COI amplicons as observed on agarose. This was further evidenced when comparing fragment sizes of Webcutter 2.0 theoretical digests (table 3) to those resolved on polyacrylamide gels.

Theoretical restriction digests of previously sequenced COI regions using Webcutter 2.0 revealed unique patterns for all species and subspecies except *D. barberi* and *D. longicornis* (table 3). Additionally, Webcutter 2.0 predicted a possible fragment pattern difference between the subspecies *D. v. virgifera* and *D. v. zeae* (table 3). However, this difference was not observed on polyacrylamide gels.

RFLP of the COI gene allowed for separation of all species assayed except for one pair of subspecies, *D. v. virgifera* and *D. v. zeae*, and one pair of sibling species, *D. barberi* and *D. longicornis*. For example, *D. barberi* polymorphs C and D were identical to *D. longicornis* polymorphs A and B. *Diabrotica barberi* haplotype A was nearly identical to *D. longicornis* polymorph C. However, there were size differences between two of the five fragments (519 vs. 619 bp and 184 vs. 180 bp) for these haplotypes. Seven of the species evaluated also exhibited diagnostic intraspecific polymorphisms: *D. barberi* (4), *D. longicornis* (3) (2 overlapping with *D. barberi*), *D. cristata* (3), *D. viridula* (2), *D. speciosa* (3), *D. u. undecimpunctata* (2), and *D. u. howardi* (2). It should also be noted that there was not a

clear geographic pattern regarding intraspecific polymorphisms as all haplotypes were observed within the same populations. Fragment pattern differences between species are treated further in the following dichotomous key developed from results shown in fig. 3.

Key to selected *Diabrotica* species found in parts of the USA, Panama and Brazil based upon AluI and MspI restriction digestion of 1308 bp of the COI gene amplified by PCR

1. Largest distinct fragment ≥ 517 bp 2
– Largest distinct fragment < 517 bp 10

2. One fragment between 396 and 344 bp .....*barberi*-A/*longicornis*-C
– No fragment between 396 and 344 bp 3

3. One fragment between 344 and 298 bp .....*barberi*-C/*longicornis*-A
– No fragment between 344 and 298 bp 4

4. One fragment between 298 and 220 bp(slightly larger than 220 bp) .....*viridula*-B
– No fragment between 298 and 220 bp 5

5. One fragment between 154 and 134 bp 6
– No fragment between 154 and 134 bp 8

6. Two fragments between 134 and 75 bp .....*barberi*-B
– One or no apparent fragments between 134 and 75 bp 7

7. Fragment between 1018 bp and 517 bp nearly equidistant from both markers .....*viridula*-A
– Fragment between 1018 bp and 517 bp closer to 517 bp marker than .....1018 bp marker .....*barberi*-D/*longicornis*-B

8. Fragment between 201 and 154 bp closer to 201 bp than 154 bp .....*u. howardi*-A
– Fragment between 201 and 154 bp closer to 154 bp than 201 bp 9

9. Excluding primer dimers, two fragments directly below 75 bp .....*u. undecimpunctata*-A
– Excluding primer dimers, one fragments directly below 75 bp .....*u. undecimpunctata*-B

10. One fragment between 344 and 298 bp 11
– No fragment between 344 and 298 bp 12

11. One fragment between 201 and 154 bp .....*cristata*-C
– No fragment between 201 and 154 bp ...*v. virgifera*/*v. zeae*

12. One fragment between 220 and 201 bp .....*porracea*
– No fragment between 220 and 201 bp 13

13. One fragment between 506 and 396 bp 14
– No fragment between 506 and 396 bp 17

14. Two fragments between 201 and 154 bp .....*balteata*
– One or no fragments between 201 and 154 bp 15

15. One or more fragments between 298 and 220 bp 16
– No fragments between 298 and 220 bp .....*cristata*-B

16. One fragment between 154 and 134 bp .....*lemniscata*
– No fragment between 154 and 134 bp .....*u. howardi*-B

17. One fragment at approximately 220 bp .....*speciosa*-A
– No fragment at approximately 220 bp 18

18. Three or more distinct fragments smaller than 75 bp .....*cristata*-A
– Less than three distinct fragments smaller than 75 bp 19

19. Five fragments between 134 and 75 bp .....*speciosa*-C
– Less than five fragments between 134 and 75 bp .....*speciosa*-B

Discussion

Results show that it is possible to distinguish between several *Diabrotica* species using PCR–RFLP of the COI gene. The use of this gene is arbitrary and it is highly probable that other mitochondrial protein coding genes can be used to differentiate these species. In preliminary studies using other mitochondrial genes such as the ND5 it was observed that many of these species could be differentiated in a similar manner (T.L. Clark, unpublished data).

While it is possible to differentiate most of the species in this study, the closely related sibling species, *D. barberi* and *D. longicornis* could not be separated. *Diabrotica barberi* and *D. longicornis* were considered to be subspecies until Krysan *et al*. (1983) separated the two species based upon morphometric characters, habitat preference and sexual isolation studies. Golden *et al*. (1992) also discriminated between adults of the two species by cuticular hydrocarbon composition analysis. Despite the separation of these sibling species, they are sympatric in part of their range (Krysan & Smith, 1987). Apparent hybrids have been collected in the field as determined by cuticular hydrocarbon analysis (L.J. Meinke, unpublished data) and hybrid progeny have been produced in the laboratory (Krysan *et al*., 1983; T.L. Clark, unpublished data). Because *D. barberi* and *D. longicornis* had the same fragment migration patterns with overlap of haplotypes, caution should be taken when using this key where these species are sympatric. However, the key should have full utility outside areas of *D. barberi* and *D. longicornis* sympatry.

The subspecies *D. v. virgifera* and *D. v. zeae* were also indistinguishable using this key. These results were not surprising as colour and incidence of *Wolbachia* induced reproductive incompatibility are the only reported characteristics that separate and support the isolation of these subspecies. In a study separating the two subspecies, Krysan *et al*. (1980) observed that elytral maculation is the best characteristic for distinguishing *D. v. virgifera* and *D. v. zeae* beetles. Behavioural studies of mating choice, mating competition and sex pheromone response revealed no significant differences between the subspecies (Krysan *et al*., 1980). Szalanski *et al*. (1999) used PCR–RFLP on 75% of the mitochondrial genome in an attempt to distinguish between the subspecies *D. v. zeae* and *D. v. virgifera*. However, their attempts were unsuccessful at revealing a diagnostic polymorphism between the two subspecies. Giordano *et al*. (1997) reported that *Wolbachia*-free *D. v. virgifera* individuals had no reproductive incompatibility with *D. v. zeae*. While these subspecies may be diverging due to a *Wolbachia* induced reproductive barrier, their divergence apparently is not ancient enough to be detected using this technique.

Despite similar fragment patterns for closely related subspecies and sibling species, intraspecific variability in

fragment patterns was observed for several species. This was expected as many *Diabrotica* exhibit intraspecific variability for several characters. For example, McDonald *et al*. (1985) showed that chromatically variable *D. barberi* adults from 15 geographic populations, ranging from Nebraska to Massachusetts, also varied in gene frequency for seven polymorphic enzymatic loci indicating possible genetic diversity between populations. Reports of geographical variation in insecticide susceptibility for *D. barberi* and *D. v. virgifera* also provide further evidence for intraspecific variability (Krysan & Sutter, 1986, Meinke *et al*., 1998). Because little is known about the genetics of many non- or minor-pest *Diabrotica*, there is little evidence of genetic variation for many of these species. Krysan & Smith (1987) reported considerable geographic colour and elytral pattern variation in *D. barberi*, *D. cristata* (Harris), *D. longicornis*, *D. lemniscata* LeConte and *D. viridula* (Fabricius) indicating the potential for intraspecific variability. The presence of intraspecific mitochondrial polymorphism in this study is yet another indicator of genetic variation. However, the biological significance and geographical patterns of mitochondrial variation for these species have yet to be established. It is also possible that more mitochondrial variation exists beyond what was presented in this study due to the limited coverage of geographic range for many of the species assayed. A series of studies that focus on individual *Diabrotica* sp. over a geographic region may provide critical information pertaining to the genetic structure and additional polymorphic markers.

In summary, a diagnostic molecular marker-based key was created for most *Diabrotica* occurring in the United States and Canada as well some common pest species that occur in Central and South America using PCR–RFLP of the COI gene. This key could be used to diagnose the *Diabrotica* complex infesting maize or other crops where multiple species have an overlapping distribution, especially in regions where insecticide resistant species overlap with susceptible pest species as occurs in some counties of Nebraska where the maize pests *D. v. virgifera* (resistant) and *D. barberi* (susceptible) are sympatric (Krysan & Smith, 1987; Meinke *et al*., 1998). The current lack of morphological identification tools (especially for larvae) and curation problems associated with enzyme studies have made such diagnoses difficult in the past (Krysan, 1986). However, information presented in this manuscript may potentially reduce these problems as alcohol preserved, dried or damaged specimens can be assayed which is advantageous over the previously described methods. If the large 1308 bp COI region used in this paper proved to be difficult to amplify via PCR, then an individual could use the COI sequences deposited in GenBank to develop PCR primers that would amplify smaller fragments which may prove to be more workable. It is our intent that this key be used as a supplement to current morphological keys to distinguish between morphologically similar species. For example, this technique could be useful in parts of Mexico, Central and South America where the majority of *Diabrotica* species occur, as the genus remains poorly collected and understudied in many of these areas. PCR–RFLP could potentially link species with previously unknown larval hosts which would most certainly aid economic, biological and evolutionary studies on *Diabrotica*.

Acknowledgements

The authors would like to thank L. Chandler (USDA-ARS), M. Green (Norvartis Corp.), S. Hanser (Zeneca Corp.), E. Vianna (EMBRAPA, Brazil),C. Hoffmann (Texas A & M University), D. Isenhour (Monsanto Corp.) and A. Sagel (Unversity of Nebraska) for their cooperation in providing specimens. We also thank Dr James Krysan (USDA-ARS, retired) who provided assistance in specimen identification. We are grateful to Mike Scharf and Ken Pruess for their critical reviews of this manuscript. This is paper is No. 12972, Journal Series, Nebraska Agricultural Division and contribution No. 1070, Department of Entomology, University of Nebraska-Lincoln.

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(Accepted 8 August 2001)