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

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

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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 AluI and MspI 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,
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 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 difficulty 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. porrecta 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 hepalid 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′-GGATCACCCTGATATAGCATTCCC-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 MgCl₂, 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.

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

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

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Fig. 1. Map of *Diabrotica* collection sites.
cycles of $94^\circ$C for 1 min, $52^\circ$C for 30 sec, $72^\circ$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$_2$O, 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 $37^\circ$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 $20^\circ$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 the 1308 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

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**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.

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

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
≥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. zae* (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. zae*, 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 ................................14
12. One fragment between 220 and 201 bp ..........porraca
    – 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 ..........lenniscata
    – 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 ..................cristata-A
19. Five fragments between 134 and 75 bp ..........speciosa-C
    – Less than five fragments between 134 and 75 bp ..............speciosa-C

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 sympathy.

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 geographical 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 curtail 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*.

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**References**


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