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GENETIC RELATIONSHIP AMONG *DIABROTICA* SPECIES (COLEOPTERA: CHRYSOMELIDAE) BASED ON RDNA AND MTDNA SEQUENCES

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ABSTRACT

Corn rootworms of the genus *Diabrotica* (Coleoptera: Chrysomelidae) are the most serious pest of corn in midwestern United States. Despite their economic importance, phylogenetic relationships within the genus remain unclear. Phylogenetic analysis of five *Diabrotica* species and subspecies was undertaken using DNA sequences of the nuclear rDNA first internal transcribed spacer region (ITS1) and a portion of the mtDNA cytochrome oxidase I and II genes (COI/COII). Parsimony and maximum likelihood analysis indicated that southern corn rootworm is sister to banded cucumber beetle, whereas, northern corn rootworm forms a distinct clade with western and Mexican corn rootworm. ITS1 and COI/COII were found to be useful markers for determining phylogenetic relationships among diabroticites.

Key Words: *Diabrotica*, rootworm, phylogenetics, mitochondrial DNA, ribosomal DNA

RESUMEN

Gusanos de raíz de maíz del genero *Diabrotica* (Coleoptera: Chrysomelidae) son la plaga de mayor seriedad para el maíz en el medio oeste de los Estados Unidos. A pesar de su importancia económica, relaciones filogenéticas dentro del genero permanecen confusas. Análisis filogenético de cinco especies de *Diabrotica* y subespecies fueron llevadas a cabo usando secuencias de ADN del [rDNA] nuclear primer orden, región [spacer] (ITS1) y una porción del [mtDNA cytochrome oxidase] I y II, genes (COI/COII). Parsimonia y [cladograms] junta-vecinos indicaron que el gusano de raíz de maíz sureño es hermano del escarabajo bandeado del pepino, mientras que los gusanos de raíz de maíz norteños forman un [clade] diferente de con los gusanos de raíz de maíz occidentales y Mexicanos. ITS1 y COI/COII resultaron ser marcadores útiles para determinar las relaciones filogenéticas entre diabroticitas.

Corn rootworms are a complex of species in the genus *Diabrotica* and are the most serious pest of corn in midwestern United States (Levine and Oloumi-Sadeghi 1991). Economically important species include western corn rootworm, *Diabrotica virgifera virgifera* LeConte (WCR); Mexican corn rootworm, *D. v. zeae* Krysan & Smith (MCR); northern corn rootworm, *D. barberi* Smith and Lawrence (NCR); banded cucumber beetle, *D. balteata* LeConte (BCB); and southern corn rootworm, *D. undecimpunctata howardi* Barber (SCR). In the United States, 20 to 25 million acres of corn are treated annually with soil insecticides to protect crops from corn rootworm larval feeding damage (Fuller et al. 1997). In addition, SCR is an economically important pest of cucurbits and peanuts, and BCB is a pest of sweet potatoes in southeastern Unites States.

Despite their importance as pest species, the phylogenetic relationships within *Diabrotica* are poorly understood, primarily because of the morphological homogeneity of the genus (Wilcox 1965, Krysan 1986). A phylogenetic study based on UPGMA clustering of allozymes of 11 Diabroticites by Krysan *et al*. (1989) supported two distinct groups, virgifera and fucata. Two molecular DNA markers, the nuclear ribosomal intergenic transcribed spacer (ITS1) and a 254 bp DNA sequence of the mtDNA NADH 4 gene have proved useful for differentiating three *Diabrotica* spp. using DNA sequences and polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP) (Szalanski and Powers 1996, Roehrdanz et al. 1998, Szalanski et al. 1999).

To date, no molecular genetic studies have resolved the phylogenetic relationships within *Diabrotica*. The goal of this study was to infer phylogenetic relationships among five economically important *Diabrotica* species and subspecies using DNA sequences of the nuclear ribosomal DNA ITS1 region and a portion of the mtDNA cytochrome oxidase I and II genes.

MATERIALS AND METHODS

Origin of specimens used in this study are listed in Table 1. Corn rootworm beetles were preserved in 70% ethanol or frozen at -20°C. Frozen voucher specimens are maintained at the USDA-ARS, Red River Valley Agricultural Research Center, Biosciences Research Laboratory, Fargo, ND.

DNA was extracted from individual legs or thoraces using Puregene DNA isolation kit D-5000A (Gentra, Minneapolis, MN) or using the high salt procedure of Cheung et al. (1993). The 3' portion of the mtDNA cytochrome oxidase (CO) I gene, tRNA leucine, and a 5' portion of the CO II gene was amplified with the primers C1-J-2797 (5'-CCTCGACGTTATTCAGATTACC-3') (Simon et al. 1994) and C2-N-3400 (5'-

TABLE 1. TAXONOMIC AND COLLECTION INFORMATION.

Sample	Origin	Date collected
Diabrotica virgifera virgifera (WCR)	Brookings Co., SD	1997
D. v. zeae (MCR)	Uvalde Co., TX	1997
D. barberi (NCR)	Howard Co., NE	1998
D. undecimpunctata howardi (SCR)	Lancaster Co., NE	1998
D. balteata (BCB)	Warton Co., TX	1999
Cerotoma trifurcata (BLB)	Lancaster Co., NE	1998
Colaspis brunnea	Lancaster Co., NE	1999

TCAATATCATTGATGACCAAT-3') (Taylor et al. 1997). The 5' ends of these primers were located at bp 2797 and 3400 of the *Drosophila yakuba* mtDNA map (Clary and Wolstenholme 1985), respectively. The 3' portion of the 18S nuclear rDNA gene, the entire ITS1 region, and the 3' region of the 5.8S gene was amplified with the primers $rDNA_2$ (5'-TTGATTACGTCCCTGCCCTTT-3', Vrain et al. 1992) and $rDNA_{1.58s}$ (5'-ACGAGCCGAGTGATCCACCG-3', Cherry et al. 1997) per Taylor and Szalanski (1999). The mtDNA PCR protocol was 35 cycles of 94°C for 45 s, 42°C for 45 s, and 72°C for 90 s. The nuclear DNA PCR protocol was 40 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 120 s. Amplified DNA from individual beetles was purified, concentrated, and sequenced per Szalanski et al. (1999). A previous study on the population genetic structure of WCR and MCR revealed a lack of genetic variation within and among populations (Szalanski et al. 1999). In NCR, ITS1 DNA sequence variation does occur (Roehrdanz et al. 1998), but at a level insufficient to influence its relationship relative to the other *Diabrotica* taxa. For the phylogenetic analysis, only one representative of WCR, MCR and NCR were obtained from Szalanski et al. (1999). GenBank accession numbers for the taxa sequenced in this study are AF195193 to AF195202.

The DNADIST program of PHYLIP v3.57C (Felsenstein 1993) was used to calculate genetic distances according to the Kimura 2-parameter (Kimura 1980) model of sequence evolution (Table 2). *Diabrotica* DNA sequences were aligned using two chrysomelids, grape colaspis *Colaspis brunnea* (Fabricius), and bean leaf beetle (BLB), *Cerotoma trifurcata* (Forster) as the outgroup taxa. Maximum likelihood and unweighted parsimony analysis on the alignments was conducted with PAUP* 4.0b2 (Swofford 1999). Gaps were treated as missing characters for all analysis. The reliability of trees was tested with a bootstrap test (Felsenstein 1985). Parsimony bootstrap analysis included 1000 resamplings using the Branch and Bound algorithm of PAUP*. For maximum likelihood analysis, the heuristic search and Hasagawa-Kishino-Yano (HKY) model of sequence evolution were used (Hasagawa et al. 1985). Par-

TABLE 2. PAIRWISE ITS1 (ABOVE DIAGONAL) AND COI/COII (BELOW DIAGONAL) DISTANCE MATRIX FOR *DIABROTICA* FROM DNADIST IN PHYLIP 3.5.

	Sample	WCR	MCR	NCR	BCB	SCR
WCR	*D. virgifera virgifera*	—	.0016	.0190	.0767	.0746
MCR	*D. v. zeae*	.0000	—	.0206	.0803	.0763
NCR	*D. barberi*	.0542	.0542	—	.0745	.0741
BCB	*D. balteata*	.1147	.1147	.1326	—	.0368
SCR	*D. undecimpunctata howardi*	.1240	.1240	.1322	.0886	—

ameters for the maximum likelihood test were Ti/Tv = 2, and (parameter of gamma distribution = 4.28 for the rDNA ITS1 sequences and 5.12 for the mtDNA sequences). For the bootstrap analysis of the maximum likelihood trees the heuristic setting was used with 100 resamplings.

Results and Discussion

The ITS1 amplicon for the five *Diabrotica* taxa ranged from 642 to 758 bp long. The mtDNA amplicon was 581 bp long for all five *Diabrotica*. The average base frequencies were A = 0.29, C = 0.17, G = 0. 21, and T = 0.33 for the entire rDNA amplicon, and A = 0.36, C = 0.15, G = 0.11, and T = 0.38 for the mtDNA amplicon. The aligned DNA data matrix, including the outgroup taxa, (available upon request, and at the web site http://ianrwww.unl.edu/ianr/plntpath/nematode/aszalans.htm) resulted in a total of

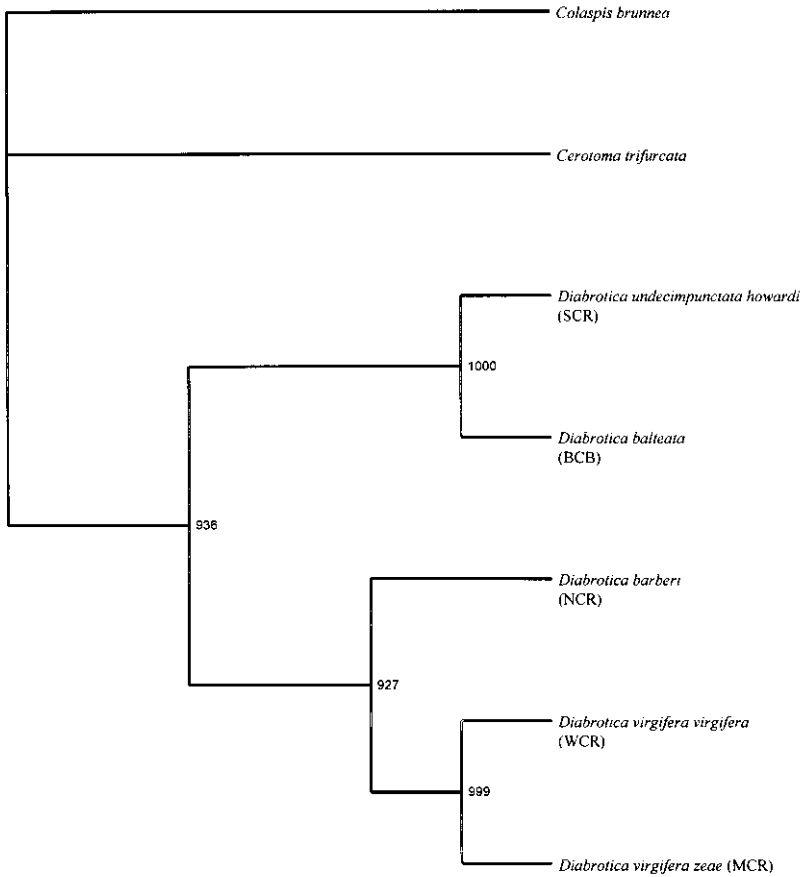

Fig. 1. A phylogenetic tree for *Diabrotica* based on DNA sequence analysis of the nuclear rDNA ITS1 region, derived from parsimony analysis and rooted by the outgroup taxa *Colaspis brunnea* and *Cerotoma trifurcata*. Bootstrap values are provided.

879 characters for ITS1 and 583 characters for COI/COII, including gaps. Of the 879 ITS1 characters, 194 characters (22%) were variable and 175 (20%) were parsimony informative characters. For the 583 COI/COII characters, 255 characters (44%) were variable and 76 (13%) characters were parsimony informative.

The rDNA dataset had only one most parsimonious tree (Fig. 1), (length = 335, CI = 0.96, CI excluding uninformative sites = 0.84). *Diabrotica undecimpunctata howardi* was depicted as the sister to *D. balteata*, with *D. barberi*, *D. virgifera virgifera*, and *D. v. zeae* representing a sister clade (Fig. 1). All of the inferred relationships were supported in >90% of the 1000 bootstrap replications. The maximum likelihood tree (-Ln likelihood = 2608.11827) yielded a phylogenetic relationship identical to the parsimony tree (Fig. 1). Parsimony (length = 360, CI = 0.89, CI excluding uninformative sites = 0.74) and maximum likelihood (-Ln likelihood = 2255.76826) analysis of the mtDNA dataset was identical to that of the ITS1 dataset (Fig. 1).

Results of the present study were congruent with those derived from allozyme and morphological data (Krysan et al. 1989, Krysan and Smith 1987). Our study supports the allozyme (Krysan et al. 1989) UPGMA phylogeny with southern corn rootworm and banded cucumber beetle forming a distinct clade (fucata group) relative to northern, western, and Mexican corn rootworm (virgifera group). The close relationship between NCR and WCR is supported by field observations of attempted interspecific mating (Krysan and Guss 1978).

This study provides a baseline for the phylogenetic relationships of this economically important genus. The ITS1 and COI/COII markers contain adequate information for phylogenetic assessment of the five *Diabrotica* studied and should prove useful for understanding the relationship of other diabroticites, and could provide the basis for species specific molecular diagnostic markers.

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