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MOLECULAR IDENTIFICATION OF THE ECONOMICALLY IMPORTANT INVASIVE CITRUS ROOT WEEVIL *DIAPREPES ABBREVIATUS* (COLEOPTERA: CURCULIONIDAE)

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Diaprepes abbreviatus L. is a polyphagous weevil affecting more than 270 species of plants. The larvae feed on roots of the trees causing damage that can kill the plant. Originally from the Caribbean, this weevil was first found in the United States in Apopka, Florida in 1964 (Woodruff 1964) and it currently infests 23 counties in Florida. In 2000, *D. abbreviatus* was discovered in the Rio Grande Valley, Texas (Skaria & French 2001) and in 2005 in southern California (California Department of Food and Agriculture, CDFA, 2007).

Different control measurements, which are specific to a particular life stage of the weevil, are currently in use (McCoy et al. 2007). However, when egg masses or larvae are found, the diagnosis of infestation is often delayed due to lack of reliable methods that allow one to identify non-adult stages. The objective of this study was to develop a method for species identification of immature stages of *D. abbreviatus* based on DNA barcoding technique. DNA barcoding consists of sequencing of a DNA segment from a specified region of the genome, and the "barcode" sequences are compared to those available in a reference database to determine the species represented by the sample. The mitochondrial gene *cytochrome oxidase I* (COI) is extensively used for barcoding of metazoans (Hebert et al. 2003). Moreover, COI sequences are the most commonly used for quarantine and forensic applications involving insects, *Tetranychus* mites (Lee & Lee 1997), *Liriomyza* leafminers (Scheffer et al. 2001) and *Calliphora* blowflies (Ames et al. 2006). In this study, a reliable method based on PCR and sequencing of the COI gene was developed to identify immature specimens of *D. abbreviatus* and to differentiate them from another common root weevil, *Pantomorus cervinus* (Boheman) Kuschel.

Eight egg-masses of *D. abbreviatus* were provided by S. Frazer at the Division of Plant Industry (DPI) (Florida Department of Agriculture and Consumer Services) rearing facility and kept at -20°C. Another clutch of eggs collected at Fairbanks Ranch (California) by D. Arena (CDFA) was reared by J. Bethke (University of California) and neonates were stored in 95% ethanol immediately upon emergence. Thirty-two live larvae were collected by digging the roots of affected trees by T. Ellis and D. Kellum (San Diego County, CA) and M.S.A. Live, quiescent, and dead adult weevils were included in the study. Larval and adult samples were preserved in 95% ethanol.

Seventy-three DNA extractions were performed with a DNeasy Tissue Kit (Qiagen, Inc.) following the manufacturer's protocol for animal tissue (Table 1). All sets of extractions were performed in parallel with 1 blank extraction (extraction negative-control: no sample). Two microliters (μL) of the DNA extraction were used to conduct PCR-amplifications of a portion of the COI gene employing the primers s1541 (5'-TGAKCYGGAATASTAGGAICATC-3'; B. Crespi, Simon Fraser University) and a2411 (5'-GCTAATCATCTAAAAACTTTAATTCCWGTWG-3'; Normark et al. 1999). All sets of PCR amplifications were performed in parallel with 1 blank reaction (PCR negative-control: no DNA). Detailed PCR-protocols can be found in Ascunce et al. (2008). Sixty-eight DNA samples were amplified successfully, and both extraction and PCR negative controls yielded no amplification products (no contamination). Five failures included DNA from adult weevils that were dead, quiescent, placed in 95% ethanol 2 h after collection or shipped in propylene-glycol. These failures were likely due to degraded DNA or the presence of PCR-inhibitors. Despite the wide range of DNA concentration values obtained among the different samples, all positive amplifications were similar in intensity assessed by visual inspection and provided similar quality sequences with the single exception of DP945.

PCR-amplifications were purified by the QIAquick PCR Purification Kit (Quiagen, Inc.) and sent to the ICBR DNA Sequencing Core at the University of Florida for sequencing. Sequences of approximately 600 base pairs (bp) were edited with Sequencher™ 3.1 (Gene Codes Corporation, Inc.) and aligned with ClustalX (Thompson et al. 1997). For the analysis of the COI sequence data, we used published *D. abbreviatus* sequences (Genbank Acc. Numbers: EF042129-EF042140) as reference sequences including the 3

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TABLE 1. DESCRIPTION OF SAMPLES, DNA EXTRACTION YIELDS AND HAPLOTYPES FOUND AFTER COMPARISONS TO REFERENCE DATABASE (ASCUNCE ET AL. 2008).

Sample ID	Collection Site[&]	Life Stage	Tissue Used[1]	Elution Buffer[2] (µL)	DNA Concentration[3] [ng/µL]	COI PCR[4]	COI-Haplotype
DP965	DPI, FL	Egg	one egg	75	1.51	+	N/S
DP970	DPI, FL	Egg	one egg	75	1.01	+	N/S
DP963	DPI, FL	Egg	egg mass	150	15.59	+	COI-1
DP964	DPI, FL	Egg	egg mass	150	107.95	+	N/S
DP966	DPI, FL	Egg	egg mass	150	45.96	+	N/S
DP967	DPI, FL	Egg	egg mass	150	0.21	+	N/S
DP968	DPI, FL	Egg	egg mass	150	28.44	+	COI-1
DP969	DPI, FL	Egg	egg mass	150	19.53	+	N/S
DP971	DPI, FL	Egg	egg mass	150	9.08	+	N/S
DP972	DPI, FL	Egg	egg mass	150	7.00	+	N/S
DP950	Fairbanks Ranch, CA	neonate	3 neonates	75	60.91	+	COI-2
DP953	Fairbanks Ranch, CA	neonate	neonate	75	21.51	+	COI-2
DP954	Fairbanks Ranch, CA	neonate	neonate	75	1.27	+	COI-2
DP955	Fairbanks Ranch, CA	neonate	neonate	75	27.31	+	COI-2
DP956	Fairbanks Ranch, CA	neonate	neonate	75	2.79	+	COI-2
DP957	Fairbanks Ranch, CA	neonate	neonate	75	0.76	+	COI-2
DP958	Fairbanks Ranch, CA	neonate	neonate	75	4.12	+	COI-2
DP959	Fairbanks Ranch, CA	neonate	neonate	75	8.38	+	COI-2
DP947	Oceanside, CA	larva	*5* mm larva	150	3.05	+	COI-2
DP948	Oceanside, CA	larva	*5* mm larva	150	246.04	+	Fuller rose beetle COI[5]
DP949	Oceanside, CA	larva	*5* mm larva	150	26.15	+	Fuller rose beetle COI[5]
DP897	Encinitas, CA	larva	*5* mm larva	150	737.74	+	COI-3
DP898	Encinitas, CA	larva	*5* mm larva	150	221.2	+	COI-3
DP899	Encinitas, CA	larva	*5* mm larva	150	226.81	+	COI-3
DP900	Encinitas, CA	larva	*5* mm larva	150	249.39	+	COI-3
DP901	Encinitas, CA	larva	5 mm larva	150	405.61	+	COI-3
DP902	Encinitas, CA	larva	5 mm larva	150	851.10	+	COI-3
DP903	Encinitas, CA	larva	5 mm larva	150	111.01	+	COI-3
DP904	Encinitas, CA	larva	5 mm larva	150	184.53	+	COI-3

[&]All collection sites in CA were in San Diego County except as indicated as [a]Los Angeles County; and [b]Orange County. [1]The amount of tissue used varied among specimens. Egg masses were irregular in shape ranging from 6 mm^2 to 1 cm^2 and were rinsed with PBS 1X buffer before extraction. One single egg or entire egg-masses were used. Larvae also varied in size and when possible a 5-8-mm segment was cut from the midsection; for small larvae the anterior portion or whole specimen was used. [2]Amount of buffer added for elution of DNA. [3]DNA concentration measured with a NanoDropTM 1000 spectrophotometer (Thermo Scientific). [4]The column "COI-PCR" indicates the presence (+) and absence (-) of PCR-amplification product. [5]Result after BLAST search: 95% similar to Fuller rose beetle COI sequence from GenBank (Accesion number: AY790876; Scataglini et al. 2005). N/S: not sequenced.

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TABLE 1. (CONTINUED) DESCRIPTION OF SAMPLES, DNA EXTRACTION YIELDS AND HAPLOTYPES FOUND AFTER COMPARISONS TO REFERENCE DATABASE (ASCUNCE ET AL. 2008).

Sample ID	Collection Site[&]	Life Stage	Tissue Used[1]	Elution Buffer[2] (µL)	DNA Concentration[3] [ng/µL]	COI PCR[4]	COI-Haplotype
DP905	Encinitas, CA	larva	5 mm larva	150	206.19	+	COI-3
DP906	Encinitas, CA	larva	5 mm larva	150	241.61	+	COI-3
DP907	Encinitas, CA	larva	5 mm larva	150	208.70	+	COI-3
DP922	Rancho Santa Fe, CA	larva	5 mm larva	150	131.92	+	COI-2
DP923	Rancho Santa Fe, CA	larva	5 mm larva	150	184.10	+	COI-2
DP924	Rancho Santa Fe, CA	larva	5 mm larva	150	349.05	+	COI-2
DP925	Rancho Santa Fe, CA	larva	5 mm larva	150	364.34	+	COI-2
DP926	Rancho Santa Fe, CA	larva	5 mm larva	150	102.74	+	Fuller rose beetle COI[5]
DP927	Rancho Santa Fe, CA	larva	5 mm larva	150	124.62	+	COI-2
DP928	Rancho Santa Fe, CA	larva	5 mm larva	150	264.35	+	COI-3
DP929	Rancho Santa Fe, CA	larva	5 mm larva	150	169.15	+	COI-3
DP930	Rancho Santa Fe, CA	larva	5 mm larva	150	403.42	+	COI-2
DP931	Rancho Santa Fe, CA	larva	5 mm larva	150	99.03	+	COI-2
DP932	Rancho Santa Fe, CA	larva	5 mm larva	150	238.27	+	COI-3
DP933	Rancho Santa Fe, CA	larva	5 mm larva	150	318.99	+	COI-3
DP934	Rancho Santa Fe, CA	larva	5 mm larva	150	175.25	+	COI-2
DP935	Rancho Santa Fe, CA	larva	5 mm larva	150	235.16	+	COI-2
DP936	Rancho Santa Fe, CA	larva	5 mm larva	150	424.7	+	COI-2
DP937	Rancho Santa Fe, CA	larva	5 mm larva	150	354.23	+	COI-2
DP938	Rancho Santa Fe, CA	larva	5 mm larva	150	263.73	+	COI-3
DP939	Rancho Santa Fe, CA	larva	5 mm larva	150	328.4	+	COI-2
DP09	DPI, FL	Adult	one leg	75	55.35	+	COI-1
DP15	DPI, FL	Adult	one leg	75	134	+	COI-2
DP18	DPI, FL	Adult	one leg	75	95.89	+	COI-1
DP22	DPI, FL	Adult	one leg	75	150.92	+	COI-1
DP24	DPI, FL	Adult	one leg	75	161.62	+	COI-1
DP859	San Diego, CA	Adult	one leg	100	10.25	+	COI-3
DP860	San Diego, CA	Adult	one leg	100	5.43	+	COI-3
DP861	San Diego, CA	Adult	one leg	100	25.13	+	COI-3
DP862	San Diego, CA	Adult	one leg	100	2.51	+	COI-3
DP863	San Diego, CA	Adult	one leg	100	23.44	+	COI-3

[&]All collection sites in CA were in San Diego County except as indicated as [a]Los Angeles County; and [b]Orange County. [1]The amount of tissue used varied among specimens. Egg masses were irregular in shape ranging from 6 mm^2 to 1 cm^2 and were rinsed with PBS 1X buffer before extraction. One single egg or entire egg-masses were used. Larvae also varied in size and when possible a 5-8-mm segment was cut from the midsection; for small larvae the anterior portion or whole specimen was used. [2]Amount of buffer added for elution of DNA. [3]DNA concentration measured with a NanoDropTM 1000 spectrophotometer (Thermo Scientific). [4]The column "COI-PCR" indicates the presence (+) and absence (-) of PCR-amplification product. [5]Result after BLAST search: 95% similar to Fuller rose beetle COI sequence from GenBank (Accesion number: AY790876; Scataglini et al. 2005). N/S: not sequenced.

TABLE 1. (CONTINUED) DESCRIPTION OF SAMPLES, DNA EXTRACTION YIELDS AND HAPLOTYPES FOUND AFTER COMPARISONS TO REFERENCE DATABASE (ASCUNCE ET AL. 2008).

Sample ID	Collection Site[&]	Life Stage	Tissue Used[1]	Elution Buffer[2] (µL)	DNA Concentration[3] [ng/µL]	COI PCR[4]	COI-Haplotype
DP864	San Diego, CA	Adult	one leg	100	23.35	+	COI-3
DP865	San Diego, CA	Adult	one leg	100	16.75	+	COI-3
DP866	San Diego, CA	Adult	one leg	100	18.67	+	COI-3
DP940	Encinitas, CA	Adult	one leg	100	12.09	—	N/S
DP941	Encinitas, CA	Adult	one leg	100	4.60	+	COI-3
DP942	Encinitas, CA	Adult-quiescent	one leg	100	1.58	—	N/S
DP960	Huntington Beach[a], CA	Adult	one leg	100	38.65	+	COI-2
DP962	Dana Point[b], CA	Adult	one leg	100	70.03	—	N/S
DP943	Carlsbad, CA	Adult-dead	head & thorax	100	4.98	—	N/S
DP944	Carlsbad, CA	Adult-dead	head & thorax	100	9.85	—	N/S
DP945	Carlsbad, CA	Adult-dead	head & thorax	100	220.55	+	failed to sequence
DP946	Carlsbad, CA	Adult-dead	two legs	100	7.26	+	COI-3
DP961	Oceanside, CA	Adult-dead	one leg	100	21.89	+	COI-2

[&]All collection sites in CA were in San Diego County except as indicated as [a]Los Angeles County; and [b]Orange County. [1]The amount of tissue used varied among specimens. Egg masses were irregular in shape ranging from 6 mm^2 to 1 cm^2 and were rinsed with PBS 1X buffer before extraction. One single egg or entire egg-masses were used. Larvae also varied in size and when possible a 5-8-mm segment was cut from the midsection; for small larvae the anterior portion or whole specimen was used. [2]Amount of buffer added for elution of DNA. [3]DNA concentration measured with a NanoDropTM 1000 spectrophotometer (Thermo Scientific). [4]The column "COI-PCR" indicates the presence (+) and absence (-) of PCR-amplification product. [5]Result after BLAST search: 95% similar to Fuller rose beetle COI sequence from GenBank (Accesion number: AY790876; Scataglini et al. 2005). N/S: not sequenced.

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mitochondrial haplotypes described for the species in the United States (Ascunce et al. 2008). All samples (eggs and adults) from the DPI colony had haplotype COI-1, with the exception of DP15, which had haplotype COI-2 (Table 1). Of the 52 sequences obtained from California samples, 23 sequences were identical to haplotype COI-2 and 26 to haplotype COI-3 (Table 1). The 3 remaining sequences obtained from larvae were different (17%) from *D. abbreviatus* sequences, but identical to each other. A BLAST search of the unknown sequence (866 bp) was performed to get an idea of what these samples may be. The highest percent similarity (95%) was obtained from the COI sequence for the Fuller rose beetle, *Pantomorus cervinus* (Accession number: AY790876). This result is not surprising since these 2 root weevils share hosts (Woodruff & Bullock 1979; McCoy et al. 2007).

Morphological description of larvae was used to differentiate 4 species of citrus weevils including *D. abbreviatus*, *P. cervinus*, *Pachnaeus opalus* Oliver, and *Pachnaeus litus* Germar (Beavers & Woodruff 1971). Accurate taxonomic identification requires highly trained personnel and it is time consuming, and morphological characters cannot identify cryptic or unknown species. Inter-specific genetic tests were described to differentiate *D. abbreviatus* and *Pachnaeus litus* egg-masses based on enzyme electrophoresis (Jones et al. 1984) and PCR of the nuclear locus coding for the *18S ribosomal RNA* gene (18S rRNA) combined with restriction fragment length polymorphisms (PCR-RFLPs) (Weathersbee et al. 2003). A shortcoming of enzyme electrophoretic techniques is that they are time consuming and the preservation of the sample is critical since biochemical markers are sensitive to degradation. One drawback of both enzyme electrophoresis and PCR-RFLP methods for the identification of unknown specimens is uncertainty of the negative results (i.e., generation of a different electrophoresis or RFLPs pattern than expected). In such cases, one can only conclude that the observable pattern is not representative of the genetic variation that the assay was designed to recognize. Another limitation of these methods is that they cannot provide data regarding the geographic origin of the weevils. Barcoding overcomes these limitations by providing sequence data that can be compared to a previously generated COI-sequence reference database for *D. abbreviatus* (Ascunce et al. 2008). The COI-haplotype composition of new infestations of immature and adult stages of *D. abbreviatus* can be monitored with this procedure. While sequencing could be expensive ($15/reaction) currently availability of high-through technology allows the process of hundreds of samples per day and lowers the costs to $2.5/sample.

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Summary

We developed a sensitive barcoding technique based on the PCR-amplification and sequencing of the mitochondrial COI gene to use in identification of eggs, larvae, and adults of the citrus root weevil, *D. abbreviatus*. This molecular tool provides accurate species identification for management and quarantine decisions of this pest.

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