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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 nonadult 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 $20^{\circ} \mathrm{C}$. 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 $(\mu \mathrm{L})$ of the DNA extraction were used to conduct PCR-amplifications of a portion of the COI gene employing the primers s1541 (5'-TGAKCYG-GAATASTAGGAICATC-3'; B. Crespi, Simon Fraser University) and a2411 (5'-GCTAAT-CATCTAAAAACTTTAATTCCWGTWG-3'; Normark et al. 1999). All sets of PCR amplifications were performed in parallel with 1 blank reaction (PCR negative-control: no DNA). Detailed PCRprotocols 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 pro-pylene-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 ${ }^{\text {TM }} 3.1$ (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: EF042129EF042140) as reference sequences including the 3
TABLE 1. DESCRIPTION OF SAMPLES, DNA EXTRACTION YIELDS AND HAPLOTYPES FOUND AFTER COMPARISONS TO REFERENCE DATABASE (ASCUNCE ET AL. 2008).

|  |  |  |  |  |  |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| Sample ID |  |  |  |  |  |

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

|  |  |  |  |  |  |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| Sample ID |  |  |  |  |  |

[^2]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 ${ }^{\text {d }}$ | Life Stage | Tissue Used ${ }^{1}$ | Elution Buffer ${ }^{2}(\mu \mathrm{~L})$ | DNA Concentration ${ }^{3}$ [ $n g / \mu \mathrm{L}$ ] | $\begin{gathered} \text { COI } \\ \text { PCR }^{4} \end{gathered}$ | 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 ${ }^{\text {a }}$, CA | Adult | one leg | 100 | 38.65 | + | COI-2 |
| DP962 | Dana Point ${ }^{\text {b }}$, 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 |

[^3]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 $18 S$ ribosomal $R N A$ gene ( 18 S rRNA) combined with restriction fragment length polymorphisms (PCRRFLPs) (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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[^1]:    ${ }^{\text {a }}$ All collection sites in CA were in San Diego County except as indicated as ${ }^{\text {a }}$ Los Angeles County; and ${ }^{\text {b }}$ Orange County. ${ }^{1}$ The amount of tissue used varied among specimens. Egg masses were irregular in shape ranging from $6 \mathrm{~mm}^{2}$ to $1 \mathrm{~cm}^{2}$ 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 $5-8-\mathrm{mm}$ 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). "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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