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Research article

A Multiplex PCR Assay for Differentiating Coconut Rhinoceros Beetle (Coleoptera: Scarabaeidae) From Oriental Flower Beetle (Coleoptera: Scarabaeidae) in Early Life Stages and Excrement

S. Watanabe and M. J. Melzer[1]

Department of Plant and Environmental Protection Sciences, College of Tropical Agriculture and Human Resources, University of Hawaii, 3190 Maile Way, St. John 205, Honolulu, HI 96822 (shizuw@hawaii.edu; melzer@hawaii.edu) and [1]Corresponding author, e-mail: melzer@hawaii.edu

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Abstract

The coconut rhinoceros beetle, *Oryctes rhinoceros* (L.), is a major pest of coconut and other palm trees. An incipient coconut rhinoceros beetle population was recently discovered on the island of Oahu, Hawaii and is currently the target of a large, mutiagency eradication program. Confounding this program is the widespread presence of another scarab beetle on Oahu, the oriental flower beetle, *Protaetia orientalis* (Gory and Percheron 1833). Eggs, early life stages, and fecal excrement of coconut rhinoceros beetle and oriental flower beetle are morphologically indistinguishable, thereby creating uncertainty when such specimens are discovered in the field. Here, we report the development of a multiplex PCR assay targeting *cytochrome oxidase I* of coconut rhinoceros beetle and oriental flower beetle that can rapidly detect and distinguish between these insects. This assay also features an internal positive control to ensure DNA of sufficient quantity and quality is used in the assay, increasing its reliability and reducing the chances of false negative results.

Key words: Coconut rhinoceros beetle, oriental flower beetle, diagnostic assay, multiplex PCR

The coconut rhinoceros beetle, *Oryctes rhinoceros* (L.), is a large scarab beetle native to Southeast Asia and a damaging pest of palm species, most notably coconut palm, *Cocos nucifera* L. Coconut rhinoceros beetle spread through much of the South Pacific in the first half of the 20th century, with current established populations in American Samoa, Fiji, Niue, Samoa, Tokelau, Tonga, and Wallis & Futuna (Catley 1969). In September 2007, coconut rhinoceros beetle was first detected on Guam in the tourist area of Tumon Bay (SPC 2007) and is now widespread across the island, where it is causing tremendous damage to coconut and phoenix palms (*Phoenix* sp.). In December 2013, adult coconut rhinoceros beetles were then found on the island of Oahu, Hawaii, at the Honolulu International Airport and the adjacent Joint Base Pearl Harbor–Hickam. Subsequent delimiting surveys revealed the presence of coconut rhinoceros beetle larvae in nearby mulch piles, representing the first documented coconut rhinoceros beetle breeding population in Hawaii. These continuing delimiting surveys, as part of a larger eradication program, indicate that coconut rhinoceros beetle occurs as discrete, incipient populations and is not widespread on Oahu.

The delimiting surveys for coconut rhinoceros beetle on Oahu include lure traps for adults as well as examination of mulch for eggs, larvae, pupae, and adults. A complicating factor in mulch examination is the presence of early life stages of another scarab insect, the oriental flower beetle, *Protaetia orientalis* (Gory and Percheron 1833). Oriental flower beetle was first reported in Hawaii in 2002 and is now widespread across the state (Leblanc et al. 2013). The eggs, first-instar larvae, and excrement of coconut rhinoceros beetle and oriental flower beetle are morphologically indistinguishable (Fig. 1). When these specimens are discovered in mulch piles outside of the currently known coconut rhinoceros beetle infestation zone, attributing them to the widespread oriental flower beetle or new infestations of coconut rhinoceros beetle can be difficult. In such cases, an incorrect identification can have considerable negative consequences. The current diagnosis of coconut rhinoceros beetle requires early life stages be reared to third instar, when an accurate morphological identification can be made (USDA-APHIS-PPQ 2014). Given the lengthy lifecycle of coconut rhinoceros beetle, it may take weeks or months to reach this life stage. For an eradication program, such a delay is impractical. Here, we report a multiplex molecular assay that can rapidly distinguish the eggs, early instars, and even fecal excrement of coconut rhinoceros beetle from oriental flower beetle, thereby providing an important tool in the coconut rhinoceros beetle response effort in Hawaii.

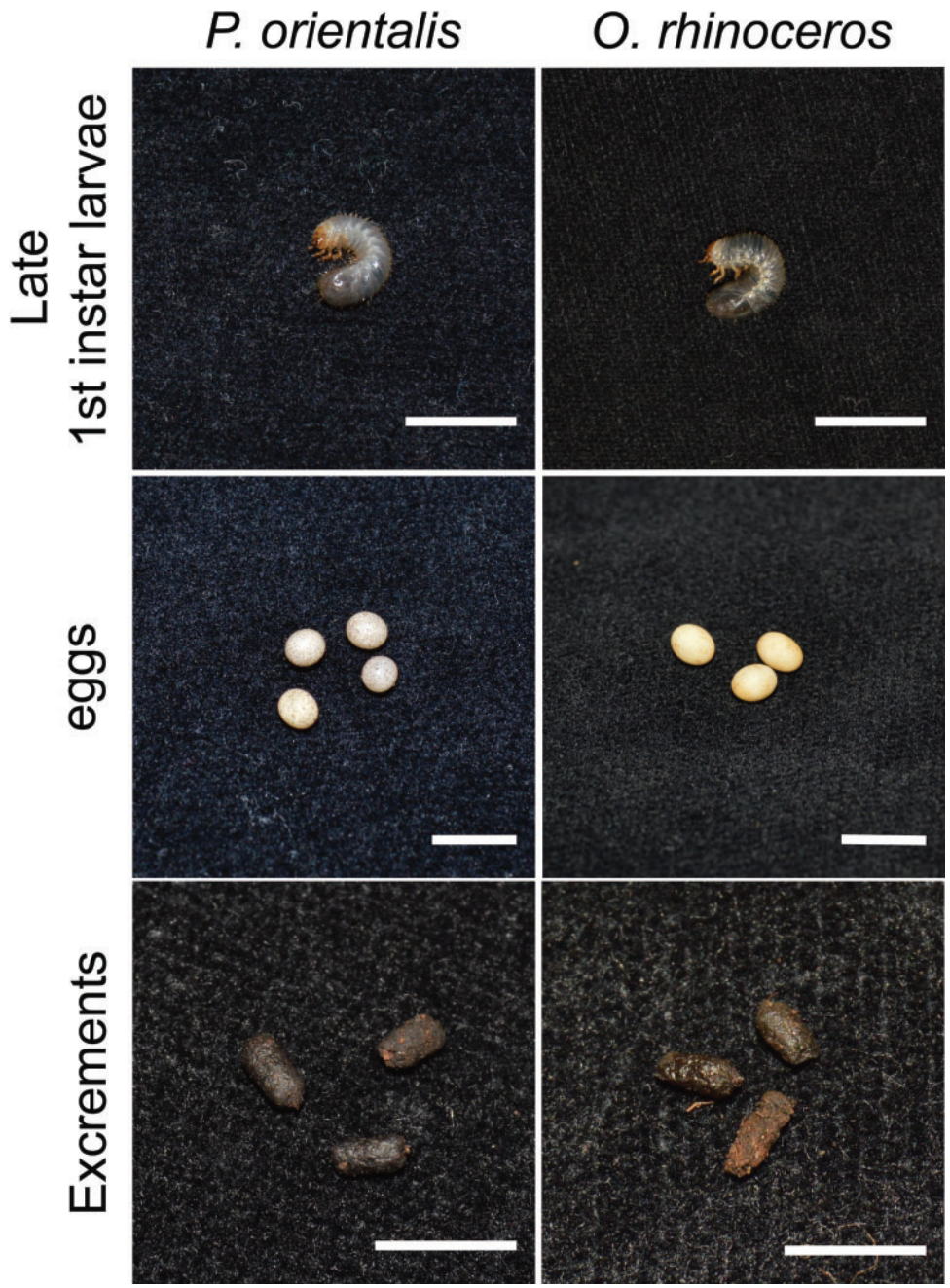

Fig. 1. Early life stages and excrement of oriental flower beetle (*P. orientalis*) and coconut rhinoceros beetle (*O. rhinoceros*) are difficult to distinguish based on morphological features. Scale bar = 5 mm.

Materials and Methods

Sample Collection

Eggs, first-instar larvae, adults, and excrement of coconut rhinoceros beetle and oriental flower beetle were collected on the island of Oahu. Additional third-instar larvae and adults of coconut rhinoceros beetle were collected from American Samoa and may represent a distinct genotype of the insect (Marshall et al. 2015, SPC 2015). Excrement samples were either immediately processed after collection from the field or stored at 4°C. Coconut rhinoceros beetle and oriental flower beetle life stages were either processed immediately or stored in either 95% ethanol or at −20 °C until processed.

DNA Extraction

DNA was extracted from coconut rhinoceros beetle and oriental flower beetle adults, larvae, and eggs using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's instructions and to a final elution volume of 100 or 150 µl. DNA was extracted from coconut rhinoceros beetle and oriental flower beetle excrement using a ZR Fecal DNA MiniPrep (Zymo Research, Irvine, CA) following the manufacturer's instructions.

Cytochrome Oxidase 1 Sequencing

The mitochondrial *cytochrome oxidase I* (*coxI*) of coconut rhinoceros beetle and oriental flower beetle was amplified by PCR

Table 1. PCR primers developed and used in this study

Primer	Sequence (5′ - 3′)	Expected product size
CRB Col f2	CCCTCTTTAACTCTACTTCTAGC	244 bp
CRB Col r2	TACTCCCCTATTTGTCTGATCTG	
OFB Col f1	GTTCTTTCAATGGGTGCTGTTTTTG	357 bp
OFB Col r1	GTGATTCCAGCTCTTTCCACCTG	
Cox f1	TTAGGTTTTATTGTATGAGCCC	104 bp
Cox r1	CCTGTWGGAACAGCAATAATTATTG	

using the universal coleopteran primers SPatR and Jerry or SJerry_F (Timmermans et al. 2010), using adult beetle DNA as template. Amplification was carried out using the following conditions: initial denaturation at 95°C for 2 min; 30 cycles of 95°C for 30 s, 51°C for 30 s, and 72°C for 1 min; and a final elongation step at 72°C for 7 min. PCR fragments (~900 bp) were resolved in 0.8% agarose gels and purified using the GenCatch Advanced PCR extraction kit (Epoch Life Science Inc., Missouri City, TX). Column-purified PCR products were ligated into a pGEM-T Easy vector (Promega, Madison, WI) and transformed into *E. coli* DH5α competent cells. At least three clones of each amplification product were sequenced at the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) facility at University of Hawaii at Manoa. Sequence data were analyzed by using FinchTV (version 1.5.0, Geospiza, Inc. Seattle, WA) and aligned with Clustal X software (www.clustal.org).

Multiplex PCR Assay

Three primer pairs were designed for the development of a multiplex PCR assay able to detect and differentiate coconut rhinoceros beetle and oriental flower beetle life stages. Specific primer pairs targeting *coxI* of coconut rhinoceros beetle (CRB CoI f2 and CRB CoI r2) and oriental flower beetle (OFB CoI f1 and OFB CoI r1) were designed based on the sequences of the nonoverlapping CRB and OFB GenBank accessions KP898260.1 and JX234248.1, respectively (Table 1). An additional primer pair, Coxf1 and Coxr1, was designed as an internal positive control (Table 1). Based on database comparisons, these primers appear to be capable of amplifying the *coxI* of many insect taxa including that of the Scarabaeidae which includes both coconut rhinoceros beetle and oriental flower beetle. This amplification product will ensure adequate DNA was extracted from different samples. Since overlapping CRB and OFB sequences were not present in GenBank, these internal positive control primers were designed based on the *coxI* sequences obtained by cloning and sequencing the PCR products generated by SPatR and Jerry or SJerry_F primers using coconut rhinoceros beetle and oriental flower beetle DNA as template.

One microliter of DNA was used as template in a 20-µl conventional multiplex PCR reaction containing GoTaq Hot Start Green Master Mix (Promega) and 10 pmol of each of the six primers. Amplification conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, 51 °C for 30 s, and 72°C for 30 s; and a final elongation step at 72°C for 7 min. Amplicons were resolved by electrophoresis in agarose gels (2% w/v) and visualized using ethidium bromide and UV light.

Results

Cytochrome oxidase 1 Sequencing

An 824-bp product was amplified from the *coxI* of both coconut rhinoceros beetle and oriental flower beetle using primers SPatR and Jerry or SJerry_F. At least three clones for each amplification product were used to generate consensus sequences which were deposited in GenBank (CRB: KX702333.1; OFB: KX702334.1). A nucleotide alignment revealed the two beetles shared 82% sequence homology over this region of *coxI* (Fig. 2).

Multiplex PCR Assay

A multiplex, conventional PCR assay was developed and evaluated for its ability to distinguish between samples containing coconut rhinoceros beetle and oriental flower beetle DNA. DNA extracted from adult, first-instar, and egg tissues of coconut rhinoceros beetle and oriental flower beetle underwent this assay, and could be clearly differentiated (Fig. 3). In addition to detecting coconut rhinoceros beetle specimens from Oahu, this assay was also able to amplify DNA from larval and adult coconut rhinoceros beetle specimens collected from American Samoa (data not shown). Furthermore, this assay also successfully determined whether excrement came from coconut rhinoceros beetle or oriental flower beetle (Fig. 3). Included in this multiplex assay were primers that amplify the coxI gene of both coconut rhinoceros beetle and oriental flower beetle. These primers serve as an internal positive control, ensuring suitable DNA was present in the assay and helping to prevent type II errors (false negative) during result interpretation. This amplification product was present in all coconut rhinoceros beetle and oriental flower beetle samples analyzed, but not present in nontemplate control reactions (Fig. 3).

When coconut rhinoceros beetle and oriental flower beetle DNA were artificially combined, three amplification products were generated (Fig. 3). This indicates the assay is also capable of identifying samples containing DNA of both coconut rhinoceros beetle and oriental flower beetle.

Discussion

Molecular assays for detecting or differentiating morphologically similar insect species are becoming increasingly common and provide a rapid approach to insect identification (Jenkins et al. 2012). For insect pests, the need to differentiate similar species may be to ensure the appropriate management strategy is undertaken (Naaum et al. 2012), or for associating a vector species with a pathogen (Garros et al. 2014). For species that require highly technical expertise for identification, molecular assays represent a reasonably straight-forward approach for identification, either as stand-alone assays (Saccaggi et al. 2008) or in parallel with morphological identification (Bahder et al. 2015). For pests of regulatory concern, rapid and accurate insect identification is essential, and molecular assays can address these needs (Dhami et al. 2016).

The ability to rapidly and reliably detect and distinguish coconut rhinoceros beetle from morphologically similar species is of utility to programs responding to this highly invasive insect. Here we report a simple, yet effective method that can use eggs, larvae, and adult beetle tissues, as well as fecal excrement that might be present in an infested area. This PCR-based assay can detect the DNA of coconut rhinoceros beetle specimens from Hawaii's incipient population, as well as specimens from American Samoa which appear to be a genetically distinct lineage of the beetle (Marshall et al. 2015). The incorporation of an internal positive control into the assay increases the reliability of the assay and reduces the chances of type II errors. This internal positive control is also theoretically capable of amplifying the *coxI* of any scarab beetle. The presence of the internal positive control product, but not the coconut rhinoceros

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Oryctes rhinoceros    ACACCCAGAGGTATATATTTTAATTCTACCTGGGTTTGGTATAATTTCACACATCATTAG 60
Protaetia orientalis  ACACCCAGAAGTTTATATTTTAATTCTCCCAGGATTTGGAATGATCTCTCACATTATTAG 60
                      ********* ** ************** ** ** ***** ** ** ** ***** *****

Oryctes rhinoceros    ACAAGAAAGAAGAAAAAAGGAAACCTTCGGAACTTTAGGAATAATCTATGCGATGATAGC 120
Protaetia orientalis  ACAGGAAAGAAGAAAAAAGGAAACATTCGGAACATTGGGGATAATTTATGCAATGATAGC 120
                      *** ******************** ******** ** ** ***** ***** ********
                                Region#1
Oryctes rhinoceros    AATTGGACTTTTAGGATTTATTGTCTGAGCCCACCATATATTTACAGTTGGTATAGACGT 180
Protaetia orientalis  AATTGGACTATTAGGTTTTATTGTATGAGCCCATCATATATTTACAGTAGGGATAGACGT 180
                      ********* ***** ******** ******* ************** ** ********
                                                                Region#2
Oryctes rhinoceros    TGATACACGAGCATACTTCACATCAGCTACAATAATTATTGCTGTTCCTACAGGAATTAA 240
Protaetia orientalis  TGATACCCGAGCTTATTTTACATCAGCAACAATAATTATTGCTGTTCCAACAGGAATTAA 240
                      ****** ***** ** ** ******** ********************* **********

Oryctes rhinoceros    AATTTTTAGATGACTAGCTACATTCCATGGGTCCCAATTAAGATACTCACCTTCTCTCTT 300
Protaetia orientalis  AATTTTTAGTTGACTTGCAACACTTCATGGTTCACAATTAAATTACTCGCCTTCTCTACT 300
                      ********* ***** ** *** * ***** ** *******  ***** ********  *

Oryctes rhinoceros    ATGATCATTAGGATTCGTATTCCTATTCACAGTGGGGGGATTAACAGGTGTAATTCTAGC 360
Protaetia orientalis  GTGAGCTTTAGGATTTGTTTTTTTATTTACAGTTGGAGGTTTAACAGGAGTTGTTTTAGC 360
                       *** * ******** ** **  **** ***** ** ** ******** **  ** ****

Oryctes rhinoceros    CAATTCATCAATTGATATTATTCTTCATGATACTTATTATGTAGTAGCCCATTTCCACTA 420
Protaetia orientalis  CAATTCTTCAATTGATATTATTTTACATGACACCTACTATGTTGTAGCTCATTTCCATTA 420
                      ****** *************** * ***** ** ** ***** ***** ******** **

Oryctes rhinoceros    CGTTTTATCAATAGGAGCAGTATTTGCCATTATAGCTGGTTTTATTCACTGATTCCCTCT 480
Protaetia orientalis  TGTTCTTTCAATGGGCGCTGTTTTTGCTATTATAGCAGGATTTGTACATTGATTTCCTTT 480
                       *** * ***** ** ** ** ***** ******** ** *** * ** ***** *** *

Oryctes rhinoceros    ATTTACAGGTCTAATAATAAACAATAAGTTCTTAAAGATCCAATTCATCACAATATTTGT 540
Protaetia orientalis  ATTCACTGGCTTAACTTTAAACAATAAGTTCTTAAAAATCCAATTTATCACAATATTTAT 540
                      *** ** **  ***   ******************** ******** ************ *

Oryctes rhinoceros    TGGAGTAAACATAACCTTTTTCCCTCAACACTTCCTTGGATTAAGAGGTATACCACGACG 600
Protaetia orientalis  TGGTGTTAACATAACTTTTTTCCCTCAACATTTTCTAGGATTAAGTGGAATACCACGACG 600
                      *** ** ******** ************** ** ** ******** ** ************

Oryctes rhinoceros    ATATTCCGACTATCCTGACGCTTACACTACATGAAACGTAATCTCATCAATTGGTTCTCT 660
Protaetia orientalis  ATACTCTGACTATCCTGATGCCTATACTACTTGAAACGTAATTTCTTCAATTGGTTCATT 660
                      *** ** *********** ** ** ***** *********** ** ***********  *

Oryctes rhinoceros    AATTTCATTAGTCAGAATTTTCATCTTTCTATTTATTATTTGAGACAGATTCGTATCAAT 720
Protaetia orientalis  AATTTCTTTAGTCAGAATTTTTATTCTATTGTTTACAATCTGAGATGCATTTGTTTCTAT 720
                      ****** ************** **  *  * ****  ** *****   *** ** ** **

Oryctes rhinoceros    ACGAAAAACACTCTCACCTCTAAGAATACCTACATCTATCGAATGAATACAAAAACTTCC 780
Protaetia orientalis  ACGAAAAACTTTATCACCTTTAAGAATAACAACGTCTATTGAGTGATTCCAGCTCTTTCC 780
                      *********  * ****** ******** * ** ***** ** *** * **     ****

Oryctes rhinoceros    CCCAGCTGAACATAGATACTCTGAACTTCCAATATTAACTAACT 824
Protaetia orientalis  ACCTGCCGAACACAGATACTCTGAACTTCCTATTCTAACTAATT 824
                       ** ** ***** ****************** **  ******* *
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Fig. 2. Alignment of the cytochrome oxidase I gene of *O. rhinoceros* and *P. orientalis*. Regions 1 and 2 indicate the annealing locations of primers Cox f1 and Cox f2, respectively.

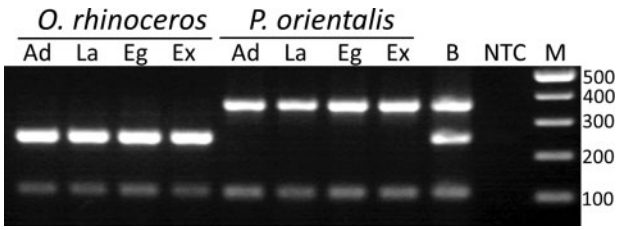

Fig. 3. Detection and discrimination of *O. rhinoceros* and *P. orientalis* using adult tissue (Ad), first-instar larva (La), eggs (Eg), and fecal excrement (Ex) using a multiplex PCR assay. B, sample containing both *O. rhinoceros* and *P. orientalis* DNA; NTC, nontemplate control; M, molecular weight marker (numbers on right indicate fragment size in base pairs). The matrix is a 2% v/w agarose gel stained with ethidium bromide and visualized with UV light.

beetle- or oriental flower beetle-specific products, would indicate that the DNA sample originated from a nontarget insect species.

The current diagnosis of coconut rhinoceros beetle requires early life stages be reared to third instar, when an accurate morphological identification can be made (USDA-APHIS-PPQ 2014). The incorporation of this rapid molecular detection and differentiation assay in Hawaii's coconut rhinoceros beetle eradication program can ensure that program resources are targeting the correct insect and eliminate this rearing period for accurate diagnosis.

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