Oryctes Nudivirus for Biocontrol of the Guam Biotype of the Coconut Rhinoceros Beetle

Aubrey Moore, University of Guam

September 2, 2017

Year: FY2016

State: Guam

Cooperative Agreement Name: Biocontrol of the Guam Biotype of the Coconut Rhinoceros Beetle

Cooperative Agreement Number: 16-8515-2058-CA

Project Funding Period: August 1, 2016 through July 30, 2017

Project Report: Farm Bill Survey Report

Project Document Date: July 12, 2016

Cooperators Project Coordinator: Aubrey Moore

Name: Aubrey Moore

Agency: College of Natural and Applied Sciences, University of Guam

Address: 303 Campus Drive

City/Address/Zip: Mangilao, Guam 96923

Telephone: (671) 735-2086

E-mail: aubreymoore@guam.net

Type of Report: Semi-Annual Accomplishment Report

Performance period covered by this report: August 1, 2016 through July 30, 2017

Contents

1 Summary

2 Objectives and Need for Assistance

The abstract from the Farm Bill Suggestion for this project provides a useful introduction:

> The population of coconut rhinoceros beetles (CRB) recently established on Guam is genetically distinct from other populations of this major palm pest and it is being referred to as the CRB-G biotype. CRB-G is resistant to Oryctes nudivirus, which is the major biocontrol agent for CRB, and it appears to have other characteristics which make it more invasive and harder to control than other CRB biotypes. While there where no range expansions of CRB for a quarter of a century (1980 to 2005), CRB is now on the move with the invasion of Guam in 2007, the Port Moresby area of Papua New Guinea in 2009, Oahu, Hawaii in 2013, and the Honiara area of Guadalcanal, Solomon Islands in 2015. It is significant that all of these new invasions involve CRB-G.
>
> This FB suggestion is a request for funding to be used as seed money to organize an international collaborative project with the goal of discovering a strain of OrNV or other microbial biocontrol agent which is highly pathogenic for CRB-Guam, to hire a graduate research assistant and to establish a insect pathology laboratory on Guam to evaluate candidate biocontrol agents discovered during foreign exploration.

2.1 Urgent Need to Mitigate Mature Palm Mortality Caused by CRB-G on Guam

Mortality of mature palms has increased dramatically over the past year as a result of abundant new breeding sites in the form of decaying vegetation left in the wake of Typhoon Dolphin which visited Guam in May 2015. It appears that the typhoon triggered a positive feedback cycle where CRB adults are numerous enough to large numbers of mature palms. The resulting dead standing coconut stems become optimum breeding sites which produce even higher numbers of adults. Uncontrolled outbreaks such as this occurred in Palau and Fiji, resulting in coconut palm mortality of 50% or more. Current tactics of trapping, sanitation, and application of Metarhizium may reduce local damage but are ineffective in preventing wide-spread island-wide damage because most breeding sites are inaccessible, in jungle and/or on military bases.

There is an urgent need to find and release an effective isolate of OrNV, or another effective density-dependent biocontrol agent, for CRB-G. Without a rapid response, most of Guam's palms may be killed and the risk of accidental transport of CRB-G to other islands is high. If CRB-G invades atolls where coconut is still "the tree of life" or islands where coconut and/or oil palms are major crops this could lead to a humanitarian disaster.

2.2 Need for Regional Collaboration to Manage CRB-G on Pacific Islands

The CRB-G biotype issue is a new emergent pest problem that has Pacific islands entomologists very worried.

- Sean Marshall presented a report entitled "A new invasive biotype of the coconut rhinoceros beetle (Oryctes rhinoceros) has escaped from biological control by Oryctes rhinoceros nudivirus" at the International Congress on Invertebrate Pathology and Microbial Control and the 48th Annual Meeting of the Society for Invertebrate Pathology in Vancouver, BC, Canada on August 13, 2015.

- The University of Guam published a press release entitled Pacific Entomologists are Worried About a New Type of Coconut Rhinoceros Beetle Discovered on Guam on September 2, 2015. This press release describes the rapidly worsening damage caused by CRB on Guam.

- Trevor Jackson published a note entitled Need for emergency response for a new variant of rhinoceros beetle (Guam biotype) in the current edition (Nov. 2015) International Association for the Plant Protection Sciences Newsletter. In this note, Jackson suggests the following steps should be taken as soon as possible to avert large scale ecological and economic damage to palms by rhino beetle invasions on Pacific islands:

    1. Raise awareness through biosecurity networks of the potential threat of CRB-Guam and provide information for early detection and eradication of limited outbreaks
    2. Form an International Working Group to develop a strategy for control and containment and coordinate activities.
    3. Identify funding sources and secure funding for key participating institutes.
    4. Carry out a thorough delimiting survey to identify current distribution of CRB-G and identify center of origin.
    5. Find and test Oryctes nudivirus variants to find CRB pathogenic strains.
    6. Implement control and containment strategy to limit impact and spread of the beetle.

- The Pacific Plant Protection Organization (PPPO) met in Fiji during the week of September 21, 2015, attended by reps from 22 Pacific Island countries and territories, the Secretariat of the Pacific Community, AgResearch New Zealand, United Nations Food and Agriculture Organization, and the United States Department of Agriculture, and federal governments of Australia and New Zealand. CRB-G was discussed at length. Jackson's suggestions were endorsed by the PPPO and the Pacific Community (SPC) was asked to assist in formulating plans and finding funding for a regional collaboration to implement these suggestions. In addition, it was suggested that "Exploration of effective biological control candidates, especially virus from the native range of the CRB-G biotype." should be a priority action item. Although success in finding an

effective OrNV isolate is not guaranteed, experts suggest there is a high probability of finding such an isolate infecting beetles near the origin of the CRB-G biotype. An endemic population of CRB-G has been found on Negros Island in the Philippines. In addition, Palau has CRB-G and other CRB biotypes. This grant will support a two weeks of field work by Moore, Marshall, and Iriarte in Palau and the Philippines. The major objective is to find an effective biocontrol agent for CRB-G and a secondary objective is to develop and test protocols for further foreign exploration.

- SPC sponsored a workshop in Fiji during June 1-3, 2016. A half-day session during this workshop was on the topic "Developing a response to the threat of CRB-G. Information exchange, development of response plans, coordination and development of new projects."

- The next opportunity for a face-to-face meeting of entomologists working on CRB-G will be at the International Congress of Entomology in Orlando, Florida during September, 2016. Many of those involved will participate in a symposium organized by Trevor Jackson and Mike Kline entitled "Scarabs without Borders: Lessons from a Century of Invasions". Plans are to use this event as an opportunity to organize a regional collaboration. This grant will support Aubrey Moore's participation in this symposium. He will make a presentation entitled "The Rhinoceros Beetle Invasion of Guam: An Unprecedented Disaster".

3 Results or Benefits Expected

- Foreign exploration leading to discovery of a highly pathogenic strain of OrNV or other microbial biocontrol agent for CRB-Guam could lead to implementation of self sustaining population suppression and tolerable damage levels on Guam.

- Loss of 50% or more of Guam's palms may be prevented if an effective biocontrol agent is found and released quickly.

- Reduction in CRB population levels on Guam will reduce the risk of accidental transport of the highly invasive CRB-Guam biotype to other Pacific islands and elsewhere An effective biocontrol agent for Guam's CRB infestation will be useful against CRB-Guam invasions elsewhere.

4 Approach

4.1 "Witch's Brew" Bioassays

In previous years, we tested several isolates of OrNV from AgResearch New Zealand and some from virus-infected beetles in Fiji. We did not observe significant mortality during many bioassays, leading us to the conclusion that CRB-G is resistant to OrNV. However, to confirm that we do not have OrNV pathogenic for CRB-G, we have started a series of "witch's brew" bioassays. Frozen, dead beetles from all previous bioassays were added to one

liter of water and made into an aqueous slurry using a blender. Vials containing remnants of virus samples from AgResearch New Zealand were agitated in 500 ml of water, and this suspension was added to the blender. The slurry was poured into a small pail and forty beetles were made to swim in this for thirty minutes. A control group of beetles was made to swim in water for thirty minutes. Beetles were kept in a large container filled with moist, commercially blended steer manure and soil. All beetles were checked weekly. Dead beetles were recorded and frozen.

We found a significantly higher mortality in beetles which swam in the slurry as opposed to beetles which swam in water. We made a fresh "witch's brew" by blending all dead beetles from this assay, and again observed mortality significantly higher than that of the control group. We will continue these witch's brew experiments and send beetle tissue samples to AgResearch New Zealand to test for OrNV.

4.1.1 Progress

Technical Support and Collaboration

- As per the work plan, a graduate student was recruited as a research assistant for this project. Ian Iriarte will fill this role as he earns a masters degree in the University of Guam's Environmental Science program. Mr. Iriarte's research topic is "Biological Control of Coconut Rhinoceros Beetle".

- A contract was prepared to facilitate collaboration between UOG and AgResearch New Zealand. Dr. Sean Marshall and Dr. Trevor Jackson, world experts on biocontrol of CRB using OrNV, work for this research center. The contract has been signed by Agresearch New Zealand and it is currently being circulated for signatures at UOG. Collabortation with colleagues at AgResearch is essential to this project because they have the skills and facilities to detect OrNV, genotype CRB, and propogate OrNV. Molecular diagnostics of a backlog of specimens in UOG freezers is awaiting completion of the contract.

Witch's Brew Experiment

- We have now completed 4 iterations of the "witch's brew" experiment. Results are summarized in table 1 and details are in tech reports available online at:

  https://github.com/aubreymoore/Witch-s-Brew/blob/master/witchesBrew1/wb1.pdf
  https://github.com/aubreymoore/Witch-s-Brew/blob/master/witchesBrew2/wb2.pdf
  https://github.com/aubreymoore/Witch-s-Brew/blob/master/witchesBrew3/wb3.pdf
  https://github.com/aubreymoore/Witch-s-Brew/blob/master/witchesBrew4/wb4.pdf

- Treatment mortality for beetles forced to swim in the "witch's brew" is high and continues to rise with each iteration. However, control mortality is high, about 30-40%, mainly due to the fungal pathogen, *Metarhizium majus*. We have attempted to filter out fungal spores by passing the brew through a serious of filters with the last stage being a Millipore 0.45 micron filter, which should block all fungal spores while allowing

Table 1: Mortality of beetles forced to swim in the witch's brew. **Treatment mortality** is corrected for control mortality using Abbott's formula. **p** is the probability of treatment mortality exceeding control mortality by chance (Fisher's exact test).

iteration	treatment mortality	p
1	51%	0.0005
2	53%	0.0014
3	82%	0.0000
4	84%	0.0000

virus particles to pass through. Unfortunately, this idea has not worked because filters get totally plugged.

- To date, we have no indication that any of the beetles which died in the "witch's brew" experiment were killed by OrNV. We are awaiting signing of the contract with Ag Research so that we can send samples to Dr. Marshall for virus detection. If any virus is detected, we will resume iterations of the "witch's brew" experiment in an attempt to increase virulence.

- We have also trapped a series of beetles an dissected out guts to send to Ag Research for virus detection. Biological control agents often arrive a few years after detection of invasive species. These "fortuitous introductions" are common. Samples will be sent to Dr. Marshall for virus detection when the AgResearch contract is signed.

4.2 Regional Collaboration on CRB-G Management

Moore will continue work with collaborators at AgResearch New Zealand and SPC to put together a regional collaboration with the objective of finding an effective biocontrol agent for CRB-G. Plans will be developed and moved forward at the scarab beetle symposium at the International Symposium of Entomology.

4.2.1 Progress

Activities at the International Congress of Entomology, Orlando, Florida

- Participated in a symposium entitled **Scarabs without Borders: Lessons from a Century of Invasions**. Abstracts for presentations relevent to CRB are available at: https://aubreymoore.github.io/CRB-G-ICE2016/Session26139.html.
  - Delivered an invited oral presentation: **The rhinoceros beetle invasion of Guam: An unprecedented disaster.** Aubrey Moore, University of Guam; Roland Quitugua, University of Guam; Trevor Jackson, AgResearch Ltd; Sean Marshall, AgResearch Ltd; Matthew Siderhurst, Eastern Mennonite University
  - Co-authored presentation: **Detection of an invasive biotype of Oryctes rhinoceros (L.) in the Pacific.** Sean Marshall, AgResearch Ltd; Maclean

Vaqalo, Secretariat of the Pacific Community; Aubrey Moore, University of Guam; Roland Quitugua, University of Guam; Trevor Jackson, AgResearch Ltd

- I helped to organized and participated in a special meeting to discuss a regional response to the coconut rhinoceros beetle biotype. This meeting was sponsored by USDA-APHIS and co-chaired by Dr. Ron Weeks and Philipp Andreozzi. Minutes from this meeting and associated data are saved in an Open Science Framework project I created at https://osf.io/67g2m/. A press release about the meeting including a photo of participants can be found here: https://osf.io/qsd8p/.

Collaboration with University of Hawaii

- On my way back from the ICE, I met with colleagues at UH-Manoa to discuss and participate in coconut rhinoceros beetle research: Mike Melzer, Shizu Watanabe, Zhiqiang Cheng, Dan Jenkins, John Allen, Hans Ramm, Mitch McLean. Discussed collaboration on development of instrumentation. Photos from a lab session available here: https://flic.kr/s/aHskK1bjRC.

4.3 Foreign Exploration for an Effective Biocontrol Agent for CRB-G

Early in 2017, Moore, Iriarte and Marshall will do field work in Palau and the Philippines. The major objective is to find an effective biocontrol agent for CRB-G and a secondary objective is to develop and test protocols for further foreign exploration. DNA analysis of CRB and OrNV will be done by AgResearch. Bioassays of any detected OrNV will be done at the University of Guam.

4.3.1 Progress

- As per the work plan, we were to visit both the Philippines and Palau during our initial foreign exploration to prospect for an OrNV isolate which can be used as an effective biocontrol agent for CRB-G. We have decided to skip a visit to Palau because these islands have been recently surveyed by our UH colleagues and we don't want to duplicate their work. Plus, we feel we have a much higher chance of finding OrNV attacking CRB-G in the Philippines which we think is within the native range of CRB-G. We will start our search on Negros Island. CRB previously collected on this island by myself and the Philippine Coconut Authority were all genotyped as CRB-G. Our trip, to include Dr. Sean Marshall, Ian Iriarte, and myself, is planned for January 23 through February 4, 2017.

5 Publications

[1] A. Moore, R. Quitugua, I. R. Iriarte, M. Melzer, S. Watanabe, Z. Cheng, and J. Muna-Barnes, "Movement of packaged soil products as a dispersal pathway for coconut rhinoceros beetle, Oryctes rhinoceros (Coleoptera:Scarabaeidae) and other invasive species," *Proceedings of the Hawaiian Entomological Society*, vol. 48, pp. 21–22, 2016. [Online]. Available: http://hdl.handle.net/10125/42743

[2] A. Moore, D. C. Barahona, K. A. Lehman, D. D. Skabeikis, I. R. Iriarte, E. B. Jang, and M. S. Siderhurst, "Judas beetles: Discovering cryptic breeding sites by radio-tracking coconut rhinoceros beetles, Oryctes rhinoceros (Coleoptera: Scarabaeidae)," *Environmental Entomology*, 2016. [Online]. Available: http://ee.oxfordjournals.org/content/early/2016/12/05/ee.nvw152

[3] S. D. G. Marshall, A. Moore, M. Vaqalo, and T. A. Jackson, "A new, virus-free haplotype of the coconut rhinoceros beetle (Oryctes rhinoceros) invades the Pacific region [IN PREPARATION]," *Journal of Invertebrate Pathology*, 2017.

6 Signatures

Dr. Lee S, Yudin, ROAR	Date
Vernon Harrington, ADODR	Date

7 Appendix 1: Report on Foreign Exploration for OrNV on Negros Island, Philippines

Molecular analysis of *Oryctes rhinoceros* collected from Philippines (January 2017)

Sean Marshall and Aubrey Moore

May 2017

Report for University of Guam

Client Report Number: 6647

agresearch

āta mātai, mātai whetū

CONTENTS

Molecular analysis of *Oryctes rhinoceros* collected from Philippines (January 2017)

1. EXECUTIVE SUMMARY

Prior to initial detection of *Oryctes rhinoceros* (coconut rhinoceros beetle; CRB) on Guam in 2007, *Oryctes rhinoceros* nudivirus (OrNV) was effective in suppressing and maintaining CRB populations at low levels on Pacific islands for over 40 years. However, to date the CRB population that has invaded Guam (CRB-G haplotype) has so far proven recalcitrant to currently available OrNV isolates that are able to cause disease in other CRB populations. OrNV is known to be widespread across the Philippines, where CRB is considered as native, and the CRB-G haplotype has been identified from here. Native habitats provide a good opportunity to identify candidate OrNV isolates (and other potential biocontrol agents).

The objective of this project was to begin searching for new OrNV isolates that could be effective against the CRB-G haplotype. Identification of an OrNV isolate with good efficacy against CRB-G would assist in preventing further mortality and damage to coconut palms on CRB-G infested Pacific islands (such as Guam), and slow or halt its spread into other areas of the Pacific region.

CRB tissue samples were obtained from live CRB adults collected using both pheromone trapping and hand collection from the Philippines. Gross observation and molecular DNA analyses were used to determine the OrNV infection status and haplotype of individual specimens. The pheromone trapping results were disappointing, with only a single CRB adult caught; however, hand collection enabled a number of adults and larvae to be collected. From the CRB specimens collected in the Philippines, one OrNV infected CRB adult was identified. This specimen was confirmed to be of the CRB-G haplotype, and OrNV from the infected tissue was isolated to prepare inoculum for testing efficacy against CRB-G.

Recommendations:

- Begin testing the new isolate as potential biocontrol agent for CRB-G.
- It is still important to continue searching for OrNV isolates (and other potential biocontrol agents) in regions known to harbor CRB-G populations, particularly where CRB caused palm damage is not reported as an issue.
- As the current CRB lure does not appear to be efficient at attracting CRB-G adults, new lures that can attract CRB-G adults need to be developed.

2. BACKGROUND

The coconut rhinoceros beetle (CRB),*Oryctes rhinoceros*, is a major pest of coconut palm, oil palm and other palm species. Palms are damaged when adult beetles bore into the crowns of palms to feed on sap. Tree mortality occurs when beetles destroy the growing tip (meristem). Immature beetles (larvae) do no damage; they feed on dead, decaying vegetation in breeding sites. Preferred breeding sites are dead, standing coconut stems, and piles of decaying vegetation such those left behind by typhoons or after replanting of oil palm plantations. If a CRB population is not suppressed, it is possible for a positive feedback cycle to initiate whereby adult beetles kill massive numbers of palms, thereby generating more food for even more grubs that turn into adults which kill even more palms. An outbreak following this scenario occurred in the Palau Islands during the late 1940s resulting in about 50% of the coconut palms being killed by CRB throughout the archipelago and 100% mortality on some of the smaller islands (Gressitt, 1953).

Following 40 years of no geographical range expansion, CRB is on the move in the Pacific. CRB has been detected for the first time at several Pacific Island locations including Saipan (2006), Guam (2007), Port Moresby, Papua New Guinea (2010), Oahu, Hawaii (2013), and Honiara, Solomon Islands (2015). Eradication of CRB is extremely difficult, having been achieved only once, on Niuatoputapu (Keppel) Island, an island with an area of only 16 km^2 belonging to the Kingdom of Tonga (Catley, 1969). Failing eradication, the usual response to CRB infestations during the second half of the 20th century was introduction of OrNV, the biological control agent of choice for this pest. OrNV infects *Oryctes rhinoceros*, typically reducing CRB damage by up to 90% with population suppression lasting indefinitely (Bedford, 2013). OrNV is auto disseminated, meaning the pathogen is carried between breeding sites by CRB adults. Like many biocontrol agents, OrNV is density dependent, working best at high population densities of CRB. Highly pathogenic OrNV isolates suppress population growth to levels, which result in only minor damage to palm species.

Current invasions of Pacific Islands by CRB involve a new invasive biotype (CRB-G) that has escaped from biological control by OrNV (Marshall et al.). Discovery of *Oryctes rhinoceros* nudivirus in the 1960s enabled the successful management of populations in Pacific Island Countries. Augmentative release of OrNV continues to be an important mechanism for CRB management in both coconut and oil palm growing regions. For 40 years after adoption of this biocontrol strategy, no new outbreaks of CRB were reported from uninfested palm growing islands in the Pacific ensuring continuity of palm based village economies. However, the situation has recently changed. For the first time in 40 years, CRB invasion into completely new areas has been reported. Additionally, Pacific areas with established CRB populations (e.g. Palau) have reported increased severity and frequency of CRB damage. Common to all these areas is the high incidence of severe palm damage not seen since the introduction of OrNV. Initial attempts to introduce OrNV into the Guam CRB population (CRB-G) were unexpectedly unsuccessful, raising the possibility that the CRB-G population that invaded Guam become tolerant or resistant to the commonly applied OrNV isolates. Analysis of several CRB populations has demonstrated that, in addition to Guam, the CRB-G biotype is also found in Hawaii, Palau, and most recently in Port Moresby (PNG) and Honiara (Solomon Islands). Within the native range of CRB, the CRB-G biotype has been detected in Taiwan, Indonesia, Malaysia, and Philippines (Marshall et al., 2017; Reil et al., 2016).

Uncontrolled infestations of CRB may kill most palms within a few years, as is currently being observed. A worse case scenario can be triggered by a massive outbreak of adult CRB emerging from abundant breeding sites caused by large amounts of decaying vegetation left in the wake of a typhoon. This is the situation in Guam, which was visited by Typhoon Dolphin in May, 2015. Very high feeding activity will kill mature coconut palms, leaving standing dead coconut trunks that are ideal breeding sites for subsequent generations of beetles. During a CRB outbreak, there will be an increased risk of further spread to uninfested islands throughout the Pacific through transportation networks. Palms are important on Pacific Islands for various reasons: as a cash crop for nuts, oil and lumber, as an ornamental tree appreciated by residents and tourists. On some of

the smaller, more traditional islands the coconut palm is referred to as the tree of life. Here, this species is an essential natural resource providing income, housing, food, oil, soap, clothing, mats, baskets, and other containers. The smaller, poorer Pacific islands will suffer the most if the spread of CRB-G cannot be controlled.

The objective of this project was to begin searching for new OrNV isolates that could be effective against the CRB-G haplotype. Native habitats provide a good opportunity to identify candidate OrNV isolates (and other potential biocontrol agents). OrNV is known to be widespread across the Philippines, where CRB is considered as native, and the CRB-G haplotype has been identified from here. Identification of an OrNV isolate with good efficacy against CRB-G would assist in preventing further mortality and damage to coconut palms on CRB-G infested Pacific islands (such as Guam), and slow or halt its spread into other areas of the Pacific region.

3. METHODS

3.1 Collection and DNA extraction of CRB tissue

CRB specimens were collected using both hand collection (larvae and adults) and 'DeFence Traps' (hanging Tekken fish gill netting) fitted with the CRB attractant, Oryctalure (www.chemtica.com). Gut tissue samples from live CRB specimens were dissected and used for DNA extraction because decaying tissue is often of limited value in terms of DNA quality. To ensure DNA quality was maintained, a 0.5 - 1 cm piece of the midgut tissue was submerged in monopropylene glycol (PPG), and stored at -20°C.

DNA was extracted from CRB tissue using a ZR Genomic DNA Tissue MiniPrep (Zymo Research) kits. DNA elution was carried out using 100 µl of elution buffer from the appropriate kit, and aliquots of eluted DNA samples were subsequently used for further analyses.

3.2 PCR-RFLP detection of CRB-G haplotype

The primers used to amplify a 523 base pair (bp) fragment of the CRB COI gene were C1-J-1718Oryctes (5'- GGAGGTTTCGGAAATTGACTTGTTCC -3') and C1-N-2191Oryctes (5'-CCAGGTAGAATTAAAATRTATACCTC -3') (Marshall et al., 2017). Each 25 µl PCR reaction constituted 0.125 µl i-StarTaq DNA Polymerase (iNtRON Biotechnology), 2.5 µl 10x PCR buffer (iNtRON Biotechnology), 0.5 µl dNTP mixture (10 mM), 0.5 µl C1-J-1718Oryctes (10 µM), 0.5 µl C1-N-2191Oryctes (10 µM), 2 µl diluted (1 in 50) DNA template, and 18.75 µl water. PCR amplifications were performed in a C2100 (BioRad) thermocycler with a cycling profile of 35 cycles of 94°C denaturation (30 s), 50°C annealing (45 s), 72°C extension (1 min) with an initial denaturation of 3 min at 94°C and a final extension of 5 min at 72°C. A 5 µl aliquot of each PCR reaction was checked by agarose gel electrophoresis (1%, 0.5xTBE), stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). For RFLP analysis (Marshall et al., 2017), successfully amplified COI PCR products (8 µl) were each combined with 0.2 µl Mse1 (10U/µl; New England BioLabs, NEB), 1 µl 10x NEB Buffer#4, 0.1 µl 100x NEB BSA and 10.7 µl water, and incubated at 37°C for 3 h. Digested samples (10 µl) were mixed with DNA loading dye, loaded onto on a 2% agarose gel in 0.5xTBE buffer. The gel was electrophoresed using 60 V for 1.5 h, stained with RedSafe and DNA fluorescence detected over UV light. Photographs were taken using an UVIdoc HD2 gel doc.

3.3 PCR detection of OrNV infection

The PCR protocol for detection of OrNV was based on that described in Richards et al. (1999), and has been subsequently modified to distinguish infection from mere presence (e.g. incidental contact contamination). The primer pairs used to amplify a 945 base pair (bp) fragment of the OrNV genome were OrNV15a (5'-ATTACGTCGTAGAGGCAATC-3') and OrNV15b (5'-ATGATCGATTCGTCTATGG-3')(Richards et al., 1999). Each 25 µl PCR reaction contained 0.2 µl i-StarTaq DNA Polymerase (iNtRON Biotechnology), 2.5 µl 10x PCR buffer (iNtRON Biotechnology), 0.5 µl dNTP mixture (10 mM), 0.5 µl OrNV15a (10 µM), 0.5 µl OrNV15b (10 µM), 1 µl diluted DNA (paired reactions of 1 in 100 and 1 in 5000), and 19.8 µl water. PCR amplifications were performed in a C2100 (BioRad) thermocycler with a cycling profile of 35 cycles of 94°C denaturation (30 s), 50°C annealing (45 s), 72°C extension (1 min) with an initial denaturation of 3 min at 94°C and a final extension of 5 min at 72°C. An 8 µl aliquot of each PCR reaction was separated by agarose gel electrophoresis (1%, 0.5xTBE), stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). Detection of OrNV PCR product in the 1 to 5000 dilution was considered indicative of OrNV infection (unpublished data; validated in combination with gross visual inspection and histological analysis based on pathology description (Huger, 2005)).

3.3 Histopathology detection of OrNV infection

Histopathology studies were performed on tissue displaying visual symptoms of OrNV infection. Dissected midgut tissue was fixed in FAA fixative (5% formaldehyde, 2.5% acetic acid, 50% ethanol as an aqueous solution) for at least 48 h. Standard paraffin embedding, serial sectioning and hematoxylin and eosin staining methods were carried out on samples by Gribbles Veterinary Services (Christchurch, New Zealand). Stained slides of the alimentary tract were examined under brightfield and differential interference contrast (DIC) optics using an Olympus BX50 upright microscope and photographed with an Olympus DP-72 digital camera.

4. RESULTS AND DISCUSSION

A total of 214 CRB larvae, pupae, and adults were collected from within the Dumagate-Dauin region of Negros Island, Philippines. All larvae were collected by hand from breeding sites. Adults were collected by three methods: by hand from breeding sites (n=39), attracted to household lighting (n=3), in pheromone traps (n=1). The single OrNV-infected beetle was caught by hand after it appeared at the illuminated house window. Pheromone traps were ineffective. Only a single beetle was caught in six traps deployed for a total of 31 trap-nights. Trap catch rate was 0.03 beetles per trap-night. One trap, which caught nothing, was deployed at a very active breeding site at a coconut sawmill.

Despite a high CRB population in the Dumaguete-Dauin region, as indicated by easily detected breeding sites and V-shaped cuts to between 20% and 40% of coconut palms, damage was very light. We saw no coconut palm mortality.

Tissue from 94 individuals was preserved for further analysis (see Appendix 1 for specimen details). Only one specimen (a hand caught adult female; Dug-42) displayed the symptoms of OrNV infection (thick, milky coloured gut; see Huger, 2005). From this collection of preserved tissue specimens, 16 representative samples (including Dug-42) were selected for molecular analysis to determine haplotype and OrNV infection status. A summary of the results is presented in Table 1.

Amplification of the partial COI gene failed for specimens Dug-55, -56, and -57; therefore, the CRB-G haplotype status could not be determined from these specimens. As COI is present in all host cells, this meant that the OrNV infection status could also not be resolved, despite the observation of OrNV DNA in specimens Dug-55 and -56. For the remaining 13 specimens, all were of the CRB-G haplotype, while only Dug-42 was positive for OrNV infection. Histopathology examination of Dug-42 gut tissue revealed diagnostic indicators of OrNV infection as described in Huger (Huger, 2005).

Since three separate methods (visual, DNA, histology) indicated OrNV presence, we conclude that the Dug-42 tissue was infected with OrNV. Isolation of OrNV from Dug-42 tissue has been undertaken, and early results from testing this material on the OrNV permissive cell line DSIR-Ha-1179 have shown the expected cytopathic effects (data not shown). If the early cell culture results are confirmed, the next step will be to conduct a pathogen challenge assay using the Dug-42 OrNV isolate against CRB-G.

Table 1: Summary of haplotype and OrNV infection status for CRB collected from Negros Island, Philippines.

Specimen[1]	COI PCR	OrNV PCR	Virus present[2]	Haplotype
Dug-1	+	-	No	CRB-G
Dug-2	+	-	No	CRB-G
Dug-16	+	-	No	CRB-G
Dug-17	+	-	No	CRB-G
Dug-18	+	-	No	CRB-G
Dug-19	+	-	No	CRB-G
Dug-42	+	+	Yes	CRB-G
Dug-43	+	-	No	CRB-G
Dug-44	+	-	No	CRB-G
Dug-45	+	-	No	CRB-G
Dug-55	-	+	Undetermined	Undetermined
Dug-56	-	+	Undetermined	Undetermined
Dug-57	-	-	Undetermined	Undetermined
Dug-62	+	-	No	CRB-G
Dug-63	+	-	No	CRB-G
Dug-69	+	-	No	CRB-G

[1] Further specimen details are listed in Appendix 1.
[2] Based on DNA analysis.

5. RECOMMENDATIONS

- Begin testing the new isolate as potential biocontrol agent for CRB-G.
- It is still important to continue searching for OrNV isolates (and other potential biocontrol agents) in regions known to harbor CRB-G populations, particularly where CRB caused palm damage is not reported as an issue.
- As the current CRB lure does not appear to be efficient at attracting CRB-G adults, new lures that can attract CRB-G adults need to be developed.

6. ACKNOWLEDGEMENTS

We thank Ian Iriarte for his assistance in collection of CRB specimens and dissection of tissue. We are also thankful for assistance from Brendan Trasmonte and Hamlet Tomaroy of the Dumaguete office of the Philippine Coconut Authority. We especially appreciated help from Bonito Ginlo who volunteered to be our local guide. This project was funded through a USDA Farm Bill grant.

7. REFERENCES

Bedford, G. O., 2013. Biology and management of palm dynastid beetles: Recent advances. Annual Review of Entomology. 58**,** 353-372.

Catley, A., 1969. The Coconut Rhinoceros Beetle *Oryctes rhinoceros* (L)[Coleoptera: Scarabaeidae: Dynastinae]. Pest Articles & News Summaries. 15**,** 18-30.

Gressitt, J. L., 1953. The Coconut Rhinoceros Beetle (*Oryctes rhinoceros*) with particular reference to the Palau Islands. Bernice P. Bishop Museum Bulletin. 212**,** 1-157.

Huger, A. M., 2005. The *Oryctes* virus: Its detection, identification, and implementation in biological control of the coconut palm rhinoceros beetle, *Oryctes rhinoceros* (Coleoptera: Scarabaeidae). Journal of Invertebrate Pathology. 89**,** 78-84.

Marshall, S. D. G., Moore, A., Vaqalo, M., Noble, A., Jackson, T. A., 2017. A new haplotype of the coconut rhinoceros beetle, *Oryctes rhinoceros*, has escaped biological control by *Oryctes rhinoceros* nudivirus and is invading Pacific islands. Journal of Invertebrate Pathology. Accepted (http://dx.doi.org/10.1016/j.jip.2017.07.006).

Reil, J. B., San Jose, M., Rubinoff, D., 2016. Low Variation in Nuclear and Mitochondrial DNA Inhibits Resolution of Invasion Pathways across the Pacific for the Coconut Rhinoceros Beetle (Scarabeidae: *Oryctes rhinoceros*). . Proceedings of the Hawaiian Entomological Society. 48**,** 57-69.

Richards, N. K., Glare, T. R., Aloali'i, I., Jackson, T. A., 1999. Primers for the detection of *Oryctes* virus from Scarabaeidae (Coleoptera). Molecular Ecology. 8**,** 1552-1553.

8. APPENDIX 1

Details of CRB tissue specimens collected from Philippines in January 2017.

Date	Specimen ID	Site	Collection Method	Stage/Gender	Condition	PPG[1]	FAA[2]	Alive[3]
26/01/2017	Dug1	Farm log2	Fallen log By hand	L3 -large	slow, reddish colour	1	1	1
26/01/2017	Dug2	Farm log2	Fallen log By hand	L3 -large	slow, red all over (almost purple)	1	1	1
26/01/2017	Dug3	Farm log2	Fallen log By hand	L3 -large	slow, reddish colour, stripped, a little purple	1	1	1
26/01/2017	Dug4	Farm log2	Fallen log By hand	L3 -large	slow, reddish colour	1	1	1
26/01/2017	Dug5	Farm log2	Fallen log By hand	L3 -small	slow, reddish colour	1	1	1
26/01/2017	Dug6	Farm log2	Fallen log By hand	L3 -small	normal	1	0	0
26/01/2017	Dug7	Farm log2	Fallen log By hand	L3 -small	normal	1	0	0
26/01/2017	Dug8	Farm log2	Fallen log By hand	L2	normal	1	0	0
26/01/2017	Dug9	Farm log2	Fallen log By hand	L2	normal	1	0	0
26/01/2017	Dug10	Farm log2	Fallen log By hand	L3 -small	normal	1	0	0
26/01/2017	Dug11	Farm log2	Fallen log By hand	L3 -small	normal	1	0	0
26/01/2017	Dug12	Farm log2	Fallen log By hand	L3 -small	normal	1	0	0
26/01/2017	Dug13	Farm log2	Fallen log By hand	L3 -small	normal	1	0	0
26/01/2017	Dug14	Farm log2	Fallen log By hand	L3 -small	normal	1	0	0
26/01/2017	Dug15	Farm log2	Fallen log By hand	L3 -small	normal	1	0	0
26/01/2017	Dug16	Lumber yard, Sibulan	Sawdust By hand	Male	vigorous, active, fatty, brown medium gut	1	1	1
26/01/2017	Dug17	Lumber yard, Sibulan	Sawdust By hand	Male	reasonabley active, not fatty, thin light brown gut	1	1	1
26/01/2017	Dugg18	Farm log1	Fallen log By hand	L3	normal	1	0	0
26/01/2017	Dugg19	Farm log1	Fallen log By hand	L3	normal	1	0	0
26/01/2017	Dugg20	Farm log1	Fallen log By hand	L3	normal	1	0	0
26/01/2017	Dugg21	Farm log1	Fallen log By hand	L3	normal	1	0	0
26/01/2017	Dugg22	Farm log1	Fallen log By hand	L3	normal	1	0	0
26/01/2017	Dugg23	Farm log1	Fallen log By hand	L3	normal	1	0	0
26/01/2017	Dugg24	Farm log1	Fallen log By hand	L3	normal	1	0	0
26/01/2017	Dugg25	Farm log1	Fallen log By hand	L3	normal	1	0	0
26/01/2017	Dugg26	Lumber yard, Sibulan	Sawdust By hand	L3 -small	flacid	1	1	1

26/01/2017	Dug27	Lumber yard, Sibulan	Sawdust By hand	L3 -small	flacid	1	1	1
26/01/2017	Dug28	Lumber yard, Sibulan	Sawdust By hand	prepupa	flacid, very fatty, lots liquid	1	1	1
26/01/2017	Dug29	Lumber yard, Sibulan	Sawdust By hand	prepupa	very fatty	1	1	1
26/01/2017	Dug30	Lumber yard, Sibulan	Sawdust By hand	L3 -large	soft flacid posterior, firm anterior (white tissue)	1	1	1
26/01/2017	Dug31	Lumber yard, Sibulan	Sawdust By hand	prepupa	very fatty	1	1	1
26/01/2017	Dug32	Lumber yard, Sibulan	Sawdust By hand	L3 -large	normal	1	1	1
26/01/2017	Dug33	Lumber yard, Sibulan	Sawdust By hand	L3 -large	normal	1	1	1
26/01/2017	Dug34	Lumber yard, Sibulan	Sawdust By hand	L3 -small	normal	1	0	0
26/01/2017	Dug35	Lumber yard, Sibulan	Sawdust By hand	L3 -large	normal	1	0	0
26/01/2017	Dug36	Lumber yard, Sibulan	Sawdust By hand	L3 -large	normal	1	0	0
26/01/2017	Dug37	Lumber yard, Sibulan	Sawdust By hand	L3 -large	normal	1	0	0
26/01/2017	Dug38	Lumber yard, Sibulan	Sawdust By hand	L3 -large	normal	1	0	0
26/01/2017	Dug39	Lumber yard, Sibulan	Sawdust By hand	L3 -small	normal	1	0	0
26/01/2017	Dug40	Lumber yard, Sibulan	Sawdust By hand	L3 -large	normal	1	0	0
26/01/2017	Dug41	Lumber yard, Sibulan	Sawdust By hand	L3 -large	normal	1	0	0
28/01/2017	Dug42	Grandma home	window by hand	Female	live, fatty, thick white gut	1	1	1
28/01/2017	Dug43	Grandma home	window by hand	Male	live, thin brown gut (kept in same bottle as #42 for 2 days)	1	1	1
28/01/2017	Dug44	Bascofadco coop, Bacong	By hand	Male	live, thin brown gut	1	1	1
28/01/2017	Dug45	Bascofadco coop, Bacong	By hand	Male	live, thin brown gut	1	1	1
28/01/2017	Dug46	Bascofadco coop, Bacong	By hand	Male	live, thin brown gut	1	1	1
30/01/2017	Dug47	Ton home, Dauin	stumps/logs By hand	Male	live, fatty, thick brown gut	1	1	1
30/01/2017	Dug48	Ton home, Dauin	stumps/logs By hand	Teneral Male	live, fatty, thick brown gut	1	1	1
30/01/2017	Dug49	Ton home, Dauin	stumps/logs By hand	Male	live, thick brown gut	1	1	1
30/01/2017	Dug50	Ton home, Dauin	stumps/logs By hand	Teneral Male	live, fatty, thick brown gut	1	0	1

30/01/2017	Dug51	Ton home, Dauin	stumps/logs By hand	Male	live, thin brown gut	1	0	1
30/01/2017	Dug52	Ton home, Dauin	stumps/logs By hand	Female	live, thin brown gut	1	0	1
30/01/2017	Dug53	Ton home, Dauin	stumps/logs By hand	Male	live, thick brown gut	1	0	1
30/01/2017	Dug54	Ton home, Dauin	stumps/logs By hand	Female	live, thin brown gut	1	0	1
30/01/2017	Dug55	Ton home, Dauin	stumps/logs By hand	L3	found dead in breeding site log	1	0	1
30/01/2017	Dug56	Ton home, Dauin	stumps/logs By hand	L2 (x6)	composite sample	1	0	1
30/01/2017	Dug57	Ton home, Dauin	stumps/logs By hand	L3	found dead in log	1	0	1
28/01/2017	Dug58	Bascofadco coop, Bacong	Fallen log by hand	L3	Healthy looking	1	0	0
28/01/2017	Dug59	Bascofadco coop, Bacong	Fallen log by hand	L3	Healthy looking	1	0	0
28/01/2017	Dug60	Bascofadco coop, Bacong	Fallen log by hand	L3	Healthy looking	1	0	0
28/01/2017	Dug61	Bascofadco coop, Bacong	Fallen log by hand	L3	Healthy looking	1	0	0
1/02/2017	Dug62	Aubrey's, Lunga	balcony light	Female	live, thin brown gut	1	1	1
1/02/2017	Dug63	Aubrey's, Lunga	Pheromone trap	Male	live, thick brown gut	1	1	1
31/01/2017	Dug64	palm plantation, San Jose	stumps/logs By hand	Female	live, thin brown gut	1	1	1
31/01/2017	Dug65	palm plantation, San Jose	stumps/logs By hand	Female	live, thin brown gut	1	1	1
31/01/2017	Dug66	palm plantation, San Jose	stumps/logs By hand	Male	live, thin brown gut, very fatty	1	1	1
31/01/2017	Dug67	palm plantation, San Jose	stumps/logs By hand	Male	live, thin brown gut	1	1	1
31/01/2017	Dug68	palm plantation, San Jose	stumps/logs By hand	Male	live, thin brown gut	1	1	1
31/01/2017	Dug69	palm plantation, San Jose	stumps/logs By hand	Female	live, thin light brown gut ,	1	1	1
31/01/2017	Dug70	palm plantation, San Jose	stumps/logs By hand	Male	live, thin brown gut, fatty	1	1	1
31/01/2017	Dug71	palm plantation, San Jose	stumps/logs By hand	Male	live, thin brown gut	1	1	1
31/01/2017	Dug72	palm plantation, San Jose	stumps/logs By hand	Female	live, thin brown gut	1	1	1
31/01/2017	Dug73	palm plantation, San Jose	stumps/logs By hand	Male	live, thin brown gut	1	1	1
31/01/2017	Dug74	palm plantation, San Jose	stumps/logs By hand	Female	live, thin brown gut	1	1	1
31/01/2017	Dug75	palm plantation, San Jose	stumps/logs By hand	Male	live, thin brown gut	1	1	1
31/01/2017	Dug76	palm plantation, San Jose	stumps/logs By hand	Male	live, medium brown gut	1	1	1

31/01/2017	Dug77	palm plantation, San Jose	stumps/logs By hand	Female	live, thin dark brown gut	1	1	1
31/01/2017	Dug78	palm plantation, San Jose	stumps/logs By hand	Female	live, thin dark brown gut	1	1	1
31/01/2017	Dug79	palm plantation, San Jose	stumps/logs By hand	Female	live, thin dark brown gut	1	1	1
31/01/2017	Dug80	palm plantation, San Jose	stumps/logs By hand	Female	live, thin brown gut	1	1	1
31/01/2017	Dug81	palm plantation, San Jose	stumps/logs By hand	Male	live, thin brown gut	1	1	1
31/01/2017	Dug82	palm plantation, San Jose	stumps/logs By hand	Female	live, thin brown gut	1	1	1
31/01/2017	Dug83	palm plantation, San Jose	stumps/logs By hand	Male	live, thin brown gut , fatty	1	1	1
1/02/2017	Dug84	Ton home, Dauin	stumps/logs By hand	Male	live, thin dark brown gut	1	1	1
1/02/2017	Dug85	Ton home, Dauin	stumps/logs By hand	Female	live, thin brown gut	1	1	1
1/02/2017	Dug86	Ton home, Dauin	stumps/logs By hand	Female	live, thin dark brown gut	1	1	1
1/02/2017	Dug87	Ton home, Dauin	stumps/logs By hand	Female	live, thin brown gut	1	1	1
1/02/2017	Dug88	Ton home, Dauin	stumps/logs By hand	Male	live, thin brown gut	1	1	1
1/02/2017	Dug89	Ton home, Dauin	stumps/logs By hand	Female	live, thin dark brown gut	1	1	1
1/02/2017	Dug90	Ton home, Dauin	stumps/logs By hand	Male	live, thin brown gut	1	1	1
1/02/2017	Dug91	Ton home, Dauin	stumps/logs By hand	Female	live, thin brown gut , fatty	1	1	1
1/02/2017	Dug92	Ton home, Dauin	stumps/logs By hand	Male	live, thin brown gut	1	1	1
1/02/2017	Dug93	Ton home, Dauin	stumps/logs By hand	Female	live, thin light brown gut	1	1	1
1/02/2017	Dug94	Ton home, Dauin	stumps/logs By hand	pupa (male)	all white (messy inards)	1	1	1

[1] 1=Tissue preserved using monopropylene glycol (PPG) for subsequent DNA analysis; 0= no PPG preservation of tissue.

[2] 1=Tissue preserved using FAA fixative (5% formaldehyde, 2.5% acetic acid, 50% ethanol as an aqueous solution) for subsequent histological analysis; 0= no FAA preservation of tissue.

[3] 1=Specimen was alive immediately prior to dissection; 0=specimen was already dead prior to dissection.

8 Appendix 2: Journal of Invertebrate Pathology Article on Discovery of CRB-G

Journal of Invertebrate Pathology 149 (2017) 127–134

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

A new haplotype of the coconut rhinoceros beetle, *Oryctes rhinoceros,* has escaped biological control by *Oryctes rhinoceros* nudivirus and is invading Pacific Islands

Sean D.G. Marshall[a,*], Aubrey Moore[b], Maclean Vaqalo[c], Alasdair Noble[a], Trevor A. Jackson[a]

[a] *AgResearch Limited, Lincoln Research Centre, Private Bag 4749, Christchurch 8140, New Zealand*
[b] *University of Guam, College of Natural and Applied Sciences, Mangilao, GU 96923, USA*
[c] *Land Resource Division, Secretariat of the Pacific Community, Suva, Fiji*

ARTICLE INFO

Keywords:
Oryctes rhinoceros
Outbreak
Population
Oryctes rhinoceros nudivirus
Tolerance
Biocontrol

ABSTRACT

The coconut rhinoceros beetle (CRB; *Oryctes rhinoceros*) is a major pest of coconut and oil palm, but the discovery and release of *Oryctes rhinoceros* nudivirus (OrNV) in the 1960s and 70s suppressed the pest such that no new invasions of uninfested islands by CRB were reported for over 30 years after implementation of the biocontrol programme. Surprisingly, a highly damaging outbreak was reported from Guam (2007), which could not be controlled by OrNV. Subsequently, new invasions have been reported from Port Moresby, Papua New Guinea (2009); O'ahu, Hawai'i (2013); and Honiara, Solomon Islands (2015). We have found that all of these outbreaks have been caused by a previously unrecognized haplotype, CRB-G, which appears to be tolerant to OrNV. PCR analysis shows that OrNV is generally present at high incidence in established populations of CRB, but is generally absent from the invasive CRB-G populations. CRB-G from Guam was not susceptible to OrNV infection by oral delivery, but injection of the virus did cause mortality. Further genetic analysis shows that CRB populations can be divided into a number of clades that coincide with the endemic and invasive history of the beetle. Analysis suggests that CRB-G originated in Asia, though the precise location remains to be discovered.

1. Introduction

Oryctes rhinoceros (Linnaeus 1758) (Coleoptera: Scarabaeidae: Dynastinae), commonly known as the coconut rhinoceros beetle (CRB), is endemic to the tropical Asia region (including South East Asia). CRB damages both coconut and oil palm, and can sometimes kill palms when adults bore into crowns to feed on sap (Bedford, 2013a, 2013b). The beetle was inadvertently introduced into the Pacific in 1909 when infested rubber tree plants were transported to Samoa from Sri Lanka (previously known as Ceylon) (Catley, 1969). The pest rapidly multiplied in Samoa and subsequently spread to several nearby Polynesian islands. Separate invasions further distributed CRB through Palau, parts of Papua New Guinea, and other Pacific nations through disruptions and uncontrolled movements during World War II (Catley, 1969; Gressitt, 1953). The invasive phase of the beetle was brought under control by the discovery and distribution of a viral biocontrol agent, *Oryctes rhinoceros* nudivirus (OrNV; previously known as *Rhabdiovirus oryctes* and *Baculovirus oryctes*). OrNV is currently present and causes persistent population suppression on many of the CRB infested Pacific Islands (Bedford, 2013b; Huger, 2005).

Virus introduction into affected Pacific Island countries and territories suppressed and weakened the CRB populations such that its spread into the Pacific islands ceased and for 30 years there was no further expansion of the range of CRB (Secretariat of the Pacific Community, 2015). Outbreaks of the beetle can still occur in conditions that provide an abundance of breeding sites, such as after cyclones or felling of mature palms for plantation replanting. The strategy for CRB management has been sanitation, coupled with population suppression using OrNV as a biocontrol (Jackson, 2009). The use of PCR in monitoring has shown that virus is regularly found in adult beetle populations, where the incidence can be over 70% (Ramle et al., 2005).

After the success of the OrNV biocontrol programme (Huger, 2005) it was surprising to see a new CRB invasion on Guam in 2007. Following a failed eradication attempt, the beetle has since spread across the whole island (Moore, personal communication). The Guam CRB population has proven to be recalcitrant to infection using commonly

Abbreviations: CRB, coconut rhinoceros beetle; OrNV, *Oryctes* nudivirus; PNG, Papua New Guinea; PoM, Port Moresby; NI, New Ireland; NB, New Britain; USDA, United States Department of Agriculture; APHIS, Animal/Plant Health Inspection Service

* Corresponding author.
E-mail address: sean.marshall@agresearch.co.nz (S.D.G. Marshall).

http://dx.doi.org/10.1016/j.jip.2017.07.006
Received 12 February 2017; Received in revised form 9 July 2017; Accepted 21 July 2017
Available online 22 July 2017
0022-2011/

applied OrNV isolates that cause disease in other CRB populations (Moore and Jackson, unpublished). Additionally, new CRB invasions have also since been reported in Port Moresby (Papua New Guinea; 2009), O'ahu (Hawai'i, USA; 2013), and Honiara (Solomon Islands; 2015).

In this paper we report efforts to control the invasive population in Guam with biocontrol and characterization of the population. We report on the identification of a new, invasive haplotype of CRB and its distribution as well as attempts to control with OrNV. The implications of a new, invasive, form of *O. rhinoceros* in the Pacific that cannot be controlled by known isolates of OrNV are discussed.

2. Materials and methods

2.1. Molecular characterization of O. rhinoceros populations

2.1.1. Collection of O. rhinoceros tissue for DNA extraction

CRB tissue samples were obtained from live CRB adults collected from Guam and several other geographic locations across the tropical Asia-Pacific region (American Samoa, Diego Garcia, Fiji, Hawai'i, India, Indonesia, Malaysia, Palau, Papua New Guinea, Philippines, Samoa, and Solomon Islands). Specimens were collected using pheromone traps baited with oryctalure (ethyl 4-methyloctanoate; ChemTica Internacional, Costa Rica). Oryctalure is an aggregation pheromone that attracts both sexes of CRB. To ensure DNA quality was maintained, a 0.5–1 cm piece of the midgut tissue from each live CRB specimen was dissected (when gut tissue dissection was not possible 2–4 legs were removed) and immediately submerged in monopropylene glycol (PPG), and stored at −20 °C until required. DNA was extracted from CRB tissue using Isolate Genomic DNA Mini (Bioline) or ZR Genomic DNA Tissue MiniPrep (Zymo Research) kits. DNA elution was carried out using 100 µl of elution buffer from the appropriate kit, and aliquots of eluted DNA samples were subsequently used for further analyses.

2.1.2. DNA sequencing of the mitochondrial COI barcode region

The 'universal barcode' primers were used to amplify a region of the *cytochrome C oxidase I* (*COI*) gene: LCO1490 (5′-GGTCAACAAATCATAAAGATATTG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994; Simon et al., 2006). Each 50 µl PCR reaction contained 0.3 µl i-StarTaq DNA Polymerase (iNtRON Biotechnology), 2.5 µl 10× PCR buffer (iNtRON Biotechnology), 0.5 µl dNTP mixture (10 mM), 0.5 µl LCO1490 (10 µM), 0.5 µl HCO2198 (10 µM), 2 µl undiluted DNA template, and 43.7 µl water. PCR amplifications were performed in a C2100 (BioRad) thermocycler with a cycling profile of 35 cycles of 94 °C denaturation (30 s), 52 °C annealing (45 s), 72 °C extension (1 min) with an initial denaturation of 3 min at 94 °C and a final extension of 5 min at 72 °C. A 5 µl aliquot of each PCR reaction was separated by agarose gel electrophoresis (1%, 0.5× TBE), stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). Successfully amplified PCR products were sent to Macrogen (www.macrogen.com/eng/) for purification and DNA sequencing. PCR amplicons were sequenced in both directions using the *COI* barcoding primers LCO1490 and HCO2198 (Folmer et al., 1994; Simon et al., 2006). Returned DNA sequences were imported into the Geneious version R8.0 software package (Kearse et al., 2012) for further sequence manipulation and analyses. Partial *COI* sequences from individual specimens were trimmed, edited, and assembled into unique contiguous sequences. The individual representative DNA sequences used have been deposited into GenBank as the following accessions: KY313828 (Malaysia-M1-1), KY313829 (PNG-NI216-1), KY313830 (PNG-ENB16-1), KY313831 (PNG-WNB16-1), KY313832 (PNG-PoM15-1), KY313833 (Malaysia-M3-2), KY313834 (India-A2), KY313835 (Malaysia-M1-10), KY313836 (Samoa-A35), KY313837 (India-A1), KY313838 (Indonesia-3), KY313839 (Palau-2), KY313840 (Palau-10), KY313841 (Indonesia-1), KY313842 (Palau-6), KY313843 (PNG-PoM15-9), KY313844 (Solomon_Islands-3), KY313845 (Philippines-Da1), KY313846 (Guam-1), KY313847 (Hawaii-1), KY313848 (PNG-PoM16-1), KY313849 (Diego_Garcia-2), KY313850 (Fiji-ViL-N1), KY313851 (Fiji-Tav-TRDC4), KY313852 (AmSamoa-15), KY313853 (Fiji-Yas-Y1), KY313854 (Samoa-A15), KY313855 (India-G2), KY313856 (Malaysia-M2-4), KY313857 (Malaysia-M3-1), KY313858 (Palau-4).

Molecular species identification used BLAST analysis (Altschul et al., 1997) of CRB *COI* sequences against the NCBI Reference Sequence Database (RefSeq Release 26) databases (O'Leary et al., 2016). Morphological species determination of Guam specimens collected during September 2007 was performed by Natalia J. Vandenburg of the USDA-ARS Systematic Entomology Laboratory. Specimens were compared with material in the US National Museum, male genitalia were dissected, and the key in Endrödi (1985) was used to determine species identity.

2.1.3. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for detecting the O. rhinoceros CRB-G haplotype

The following primer pair was designed and used to amplify a 523 bp fragment of the CRB *COI* gene: C1-J-1718Oryctes (5′-GGAGGTTTCGGAAATTGACTTGTTCC-3′) and C1-N-2191Oryctes (5′-CCAGGTAGAATTAAAATRTATACCTC-3′). A unique *Mse*I restriction site polymorphism within this amplified region allows the CRB-G haplotype to be identified. Each 25 µl PCR reaction contained: 0.2 µl i-StarTaq DNA Polymerase (iNtRON Biotechnology), 2.5 µl 10× PCR buffer (iNtRON Biotechnology), 0.5 µl dNTP mixture (2.5 mM each), 0.5 µl C1-J-1718Oryctes (10 µM), 0.5 µl C1-N-2191Oryctes (10 µM), 1 µl undiluted CRB DNA template, and 19.8 µl water. PCR amplifications were performed in a C2100 thermocycler (BioRad) with a cycling profile of 35 cycles of 94 °C denaturation (30 s), 50 °C annealing (45 s), 72 °C extension (1 min) with an initial denaturation of 3 min at 94 °C and a final extension of 5 min at 72 °C. A 5 µl aliquot of each PCR reaction was checked by agarose gel electrophoresis (1%, 0.5× TBE), stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). For RFLP analysis, successfully amplified *COI* PCR products (8 µl) were each combined with 0.2 µl Mse1 (10U/µl; New England BioLabs, NEB), 1 µl 10× NEB Buffer#4, 0.1 µl 100× NEB BSA and 5.7 µl water, and incubated at 37 °C for 3 h. Digested samples (15 µl) were mixed with DNA loading dye and loaded onto on a 2% agarose gel in 0.5 × TBE buffer. The gel was electrophoresed using 60 V for 1.5 h, stained with RedSafe dye, and DNA fluorescence detected over UV light. Photographs were taken using an UVIdoc HD2 gel doc. The DNA fragment sizes obtained following the *Mse*I digest are shown in Fig. 1.

2.1.4. Phylogenetic analysis of the O. rhinoceros COI barcode region

Assembled CRB *COI* barcode sequences were aligned using the MUSCLE algorithm (default parameters) as implemented within Geneious R8.0. After removal of redundant sequences from the alignment, a dataset of 31 geographically representative sequences remained. Further trimming of the alignment was done to minimize end gaps, which yielded a 676 bp sequence fragment from of the *COI* gene. Tree construction was inferred from Bayesian phylogenetic analysis using an HKY85 model with a Gamma rate variation setting carried out in Geneious R8.0. Posterior probabilities were calculated over 2.0×10^6 generations.

2.2. Pathogen challenge bioassay

2.2.1. Collection, rearing, and maintenance of adult O. rhinoceros

Live adult *O. rhinoceros* were field collected from Guam using pheromone traps baited with oryctalure (ethyl 4-methyloctanoate; ChemTica Internacional, Costa Rica). In the lab individual beetles were

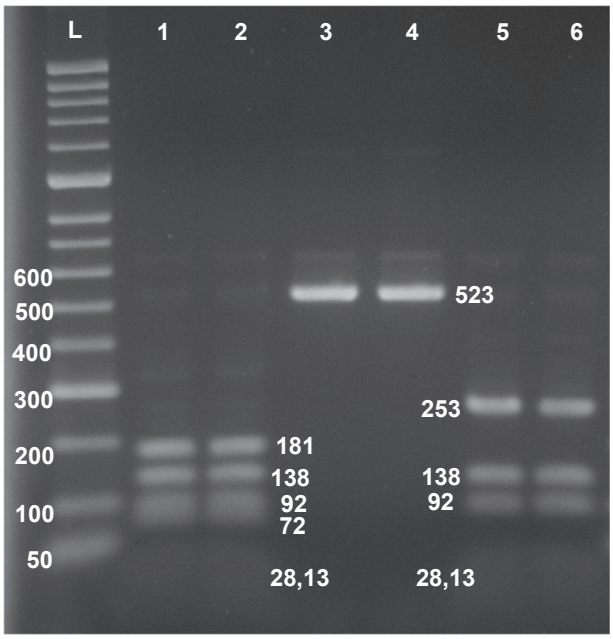

Fig. 1. Exemplar PCR-RFLP analysis results that distinguish the CRB-G haplotype from other CRB populations. Results shown are examples of CRB-S (lanes 1–3) and CRB-G (lanes 4–6) haplotype specimens. Following PCR amplification of a 523 bp fragment of the *COI* gene from *O. rhinoceros* DNA, the amplicons were digested with the restriction enzyme *Mse*I and the generated fragments subsequently separated on a 2% agarose gel. The ladder lane corresponds to a 100-bp DNA size ladder, lanes 1, 2, 5, and 6 are *Mse*I-digested CRB PCR amplicons from independent CRB specimens, and lanes 3 and 4 exemplify undigested PCR amplicons. Numbers on the figure next to the DNA bands indicate fragment size in base pairs.

incubated at 25 °C in individually labelled 500 ml glass Mason jars containing moist peat moss. The top of each jar was enclosed with a metal disk that had a single hole punched in it. A piece of cloth or paper towel was trapped between the top of the disk and the screw ring to prevent small insects from entering or leaving the jars. Slices of banana were provided as food and replenished as required.

2.2.2. Preparation of *O. rhinoceros nudivirus (OrNV) isolates for inoculation of O. rhinoceros*

The following OrNV isolates were used in the pathogen challenge assays: OrNV-X2B (commonly used within the Pacific region for augmentative release; isolated from Bugsuk Island, Philippines); OrNV-I (isolated from Kerala, India), OrNV-TAS (isolated from Upolo, Samoa), OrNV-TAP (isolated from Savaii, Samoa), and OrNV–MalB (isolated from Perak, Malaysia). The OrNV-MalB isolate was extracted from fresh gut tissue (supernatant from macerate passed through a 0.22 µm filter to sterilize) that had been confirmed to be the correct isolate (Crawford et al., 1986; Ramle et al., 2005). The OrNV-X2B, -I, -TAS, and -TAP were previously isolated and propagated using cell culture methods as described by Crawford and Sheehan (1984). Briefly, cells of the *Heteronychus arator* DSIR-Ha-1179 cell line (BB) (Crawford, 1982) were seeded into culture flasks and grown in PS100 medium (Grace's insect cell medium (Sigma), 2.95 g/l tryptose phosphate broth (Sigma), 1 ml/l TC-100 vitamins with the pH adjusted to 6.2 (using potassium hydroxide) and further supplemented with fetal bovine serum to 10% (Life Technologies) and gentamicin (25 µg/ml) (Sigma)). Culture flasks were incubated in air at 27°C. When the cell culture reached 25% confluence, OrNV isolates from sterile stocks were inoculated into appropriate flasks that were incubated for a further 10–14 days to allow virus multiplication. Virus was harvested by centrifugation of resuspended flask contents to obtain an OrNV containing cell-free supernatant. OrNV titer was quantified as infectious units per milliliter (IU/ml) by end-point dilution analysis as previously described (Pushparajan et al., 2013).

2.2.3. Bioassay treatments

Oral treatments of adult *O. rhinoceros* were carried out using a modification of standard methods previously described (Lacey, 2012; Zelazny, 1978). Oral treatments of OrNV isolates for dosing field collected *O. rhinoceros* adults were administered as two sequential 30 µl doses of virus (5×10^4 IU of virus per dose prepared in a sterile 10% (w/v) sucrose solution). A control treatment (sterile 10% (w/v) sucrose solution) was also included. The first dose involved immobilizing adults on their backs, applying a droplet of solution directly onto their mouth parts, and allowing the full dose to be consumed before placing each beetle individually into an empty container. The following day a second dose was absorbed into a slice of banana and placed into the container for consumption over 3–4 more days. Following this, moist peat moss was placed into the container, and fresh untreated banana slices were provided as food and replenished as required. CRB were inspected for symptoms of OrNV infection (reduced feeding, lethargy, mortality) at regular intervals over eight weeks, and observations recorded. On completion of the experiment, or after death, beetles were dissected for visual evidence of disease and gut tissue removed for PCR and histological examination.

Direct hemocoel treatments of adult *O. rhinoceros* were carried out using a modification of methods previously described (Lacey, 2012; Zelazny, 1978). To prevent accidental infection arising from the injection process, beetles were surface sterilize with 70% ethanol prior to inserting needle through the cuticle (junction of hind leg and body) parallel to the gut to avoid puncture. A single 30 µl treatment dose of sterile virus (5×10^4 IU in sterile PS100 medium) or control (sterile PS100 medium containing no virus) was provided using sterile 1 ml syringes fitted with 30-gauge needles. Treated insects were placed in individual containers and monitored over two days for signs of mortality caused by the injection process. Following this period, moist peat moss was added, and fresh untreated banana slices were provided as food and replenished as required. Assessments were made as above.

Bioassays were carried out as maximum challenge tests, with both oral, hemoceol and control treatments set up over two separate days on two different occasions. Maximum dose pathogen challenge bioassays were carried out using a range of isolates; X2B, TAS and TAP (isolated from Samoa), I (isolated from Kerala, India), and MalB (isolated from Perak, Malaysia). Observations were recorded at regular intervals over eight weeks to detect symptoms of OrNV infection (reduced feeding, lethargy, mortality). The data were analyzed with a generalized linear model for the proportion dead and the treatments as a factor using a binomial distribution as implemented by R software (R Core Team, 2017). Separate models were fitted to the data for the oral and hemocoel routes.

2.3. Diagnosis of *O. rhinoceros nudivirus infection*

2.3.1. PCR detection of *O. rhinoceros nudivirus infected O. rhinoceros beetles*

CRB gut tissue dissected from moribund or dead bioassay specimens had DNA extracted as described in Section 2.1.1. The PCR protocol for detection of OrNV was based on that described in Richards et al. (1999), and has been subsequently modified by using diluted DNA template (down to 1 in 5000) to better distinguish infection from mere presence (e.g. incidental contact contamination). The primer pairs used to amplify a 945 base pair (bp) fragment of the OrNV genome were OrNV15a (5′-ATTACGTCGTAGAGGCAATC-3′) and OrNV15 b (5′-ATGATCGATTCGTCTATGG-3′) (Richards et al., 1999). Each 25 µl PCR reaction contained 0.2 µl i-StarTaq DNA Polymerase (iNtRON Biotechnology), 2.5 µl 10× PCR buffer (iNtRON Biotechnology), 0.5 µl dNTP mixture (10 mM), 0.5 µl OrNV15a (10 µM), 0.5 µl OrNV15 b (10 µM), 1 µl diluted DNA (paired reactions of 1 in 100 and 1 in 5000), and 19.8 µl water. PCR amplifications were performed in a C2100

(BioRad) thermocycler with a cycling profile of 35 cycles of 94 °C denaturation (30 s), 50 °C annealing (45 s), 72 °C extension (1 min) with an initial denaturation of 3 min at 94 °C and a final extension of 5 min at 72 °C. An 8 µl aliquot of each PCR reaction was separated by agarose gel electrophoresis (1%, 0.5× TBE), stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). Detection of OrNV PCR product from the 1 in 5000 dilution was considered indicative of OrNV infection and has been validated (unpublished) by comparison with pathological effects such as gross visual inspection and histological analysis based on diagnostics described by Huger (2005).

2.3.2. Visual and histological observations of field collected and O. rhinoceros nudivirus challenged O. rhinoceros beetles

When taking gut samples (Section 2.1.1) from field collected or bioassay challenged beetles, a visual diagnosis was carried out of gut condition to look for evidence of gut swelling and whitening typical of OrNV infection (Huger, 2005). In addition to gut samples for PCR, a subset of samples from field collected and moribund bioassay specimens was taken for histology. Samples were immersed for 48 h in FAA fixative (5% formaldehyde, 2.5% acetic acid, 50% ethanol as an aqueous solution) before paraffin embedding, serial sectioning, and hematoxylin and eosin (H & E) staining (Kiernan, 1990). Slides of gut tissue were examined under bright-field and differential interference contrast (DIC) optics with observations of OrNV infection status recorded based on pathology described by Huger (2005).

3. Results and discussion

3.1. Characterisation of the CRB Guam population and attempts to introduce OrNV

In 2007, beetles collected from Guam were recognized as *Oryctes rhinoceros*, based on morphological characteristics and damage to coconut palms (Berringer, 2007); there is no record of CRB from Guam prior to 2007 (Moore, personal communication). To confirm the invasive beetle present in Guam was in fact *O. rhinoceros* (as found in other Pacific regions) the universal barcoding region of the *COI* gene (bases1490-2198) was PCR amplified and DNA sequenced. Sequences from all ten of the initial Guam specimens analyzed were identical across this gene region. When compared against DNA sequences obtained from other CRB specimens collected within Fiji, PNG, and Samoa, a level of less than 2% DNA sequence variation was observed within this region. As this fell within the accepted < 2 to 3% level of difference for a single species (Hebert et al., 2003a, 2003b; Meyer and Paulay, 2005), it validated the original supposition that the invasive Guam beetle population was species *O. rhinoceros*. Subsequent comparative morphological analysis by the USDA-ARS Systematic Entomology Laboratory (unpublished) further confirmed the insect identification *as O. rhinoceros.*

Following the successful use of using OrNV as a biocontrol agent to suppress CRB populations within the Pacific region (Huger, 2005), establishment of virus disease was attempted in Guam. However, attempts to infect CRB from Guam with the OrNV X2B isolate (commonly used in the Pacific region in augmentative release biocontrol programmes; isolated from Bugsuk Island, Philippines) were unexpectedly unsuccessful (Moore and Jackson, unpublished). Bioassays were repeated as maximum challenge tests using a range of isolates, with treatment doses delivered through either oral or hemocoelic routes. Lack of impact of the virus on the Guam beetles was confirmed with no observed differential effect from the control on feeding or morbidity. Mortality results from the bioassay are presented in Table 1 (corrected mortality values are presented in Supplementary Table S1). Mortality following oral dosing with OrNV isolates ranged from 28.6% to 62.5%, with none of the isolates tested producing mortality statistically significantly different to the control mortality (28.1%). After hemocoelic injection, mortality ranged 62.5–93.3%, with all four isolates tested producing statistically significant mortality ($p < 0.05$) compared with the control (30.4%). On beetle death or at completion of the experiments beetles were dissected for visual diagnosis of the white and swollen midgut symptomatic of OrNV infection (Huger, 2005). There was no substantive visual evidence that the virus treated beetles were infected as most (> 98%) had normal coloured unswollen midguts. From a subset of 17 specimens analyzed by histopathology and PCR, signs of OrNV were detected in a total of nine specimens, with two out of nine found from orally dosed beetles (none with white swollen midguts), and seven out of eight identified dosed via the hemocoel route (three with white swollen midguts). These results are consistent with field observations as white and swollen midgut symptoms typical of OrNV infection have never been observed in wild-caught CRB from Guam despite virus treated beetle release, while they are routinely observed in susceptible populations of CRB from locations where OrNV is present (e.g. Fiji Malaysia, Samoa).

Table 1
O. rhinoceros mortality data from OrNV pathogen challenge assays.

Route	Treatment	n =	# Dead	Mortality (%)[a]	SE (%)[b]	*p*-value[c]
Oral	Control	32	9	28.1	7.9	
	OrNV-I	14	4	28.6	12.1	0.975
	OrNV-X2B	22	11	45.7	7.3	0.106
	OrNV-TAS	10	4	40.0	15.5	0.081
	OrNV-TAP	8	5	62.5	17.1	0.481
	OrNV-MalB	46	21	50.0	10.7	0.121
Hemocoel	Control	23	7	30.4	9.6	
	OrNV-I	23	15	65.2	9.9	0.021*
	OrNV-X2B	24	15	70.0	14.5	0.031*
	OrNV-TAS	15	14	62.5	9.9	0.043*
	OrNV-TAP	10	7	93.3	6.4	0.002*
	OrNV-MalB	NA				

[a] Proportion of mortality from observations.
[b] SE, standard error of the proportional mortality.
[c] *p*-values based on comparison of the control treatment to each of the OrNV treatments. *p*-values were calculated using a generalized linear model for the proportion dead and the treatments as a factor using a binomial distribution.
* Indicates significance at a > 95% confidence level.

Failure to produce significant levels of mortality from oral inoculation in the Guam CRB was surprising. OrNV has been widely used as a biocontrol agent (Huger, 2005). This is based on well-established evidence of its infection and pathogenicity, observations of reduced beetle populations and palm damage, and ease of establishment (Bedford, 2013a; Jacob, 1996; Jayawardena, 2013; Zelazny, 1973, 1979). Additionally, in recent concurrent tests using OrNV produced by cell culture, the virus was proven to be pathogenic (> 90% mortality by eight weeks after treatment) to a Malaysian population of CRB (Khudri et al., 2016). As the natural route of OrNV infection for CRB is oral ingestion (Huger, 1966; Zelazny, 1976), these observations strongly suggested that the Guam population is highly tolerant (if not completely resistant) to oral infection by the OrNV isolates tested.

3.2. CRB-G haplotype identification and distribution

Due to the unexpected difficulties in establishing *per os* OrNV infection within the Guam CRB population, the partial *COI* sequences were inspected for variable sites that could possibly distinguish it from the archetypal OrNV-susceptible CRB populations found elsewhere. A fixed base change was found to exclusively correlate with the Guam CRB population. This was located at nucleotide position 288 within the 676 bp sequence fragment examined. An A > G transition (nucleotide position 288) was centered on a *Mse*I restriction site. From this observation, a PCR-RFLP assay was developed and validated, which enabled populations related to the Guam CRB invasion (referred to as the CRB-G haplotype) to be distinguished from other CRB populations.

Table 2
Summary of *O. rhinoceros* haplotype and OrNV presence by location.

Location[a]	Haplotype[e]	OrNV present[f]	n = [g]	% CRB-G	% OrNV +
American Samoa	**CRB-S**	**No**	**2**	**0**	**0**
Tutuila	CRB-S	No	2	0	0
Diego Garcia	***CRB-S***	***ND***	***2***	***0***	***ND***
Fiji	***CRB-S***	***Yes***	***34***	***0***	***47.1***
Viti Levu	CRB-S	Yes	21	0	61.9
Vanua Levu	CRB-S	Yes	10	0	10
Yasawa	CRB-S	Yes	3	0	33.3
Guam	***CRB-G***	***No***	***17***	***100***	***0***
Hawai'i	***CRB-G***	***No***	***14***	***100***	***0***
O'ahu	CRB-G	No	14	100	0
India	***CRB-S***	***Yes***	***4***	***0***	***50***
Kerala	CRB-S	Yes	4	0	50
Indonesia	***CRB-G, CRB-S***	***No***	***7***	***57.1***	***0***
Sumatra	CRB-G, CRB-S	No	7	57.1	0
Malaysia[b]	***CRB-S***	***Yes***	***31***	***0***	***45.2***
Type A OrNV[b]	CRB-S	Yes	24	0	45.8
Type B OrNV[b]	CRB-S	Yes	7	0	42.9
Palau[c]	***CRB-S, CRB-G***	***Yes (CRB-S, -G)***	***11***	***72.7***	***72.7***
Aimeliik	CRB-S, CRB-G	Yes (CRB-S, -G)	8	62.5	75
Ngarraard	CRB-G	No	1	100	0
Airai	CRB-G	Yes (CRB-G)	2	100	100
Papua New Guinea[d]	***CRB-S, CRB-G***	***Yes (only CRB-S)***	***143***	***6.3***	***49***
New Ireland	CRB-S	Yes	86	0	62.8
West New Britain	CRB-S	Yes	31	0	22.6
East New Britain	CRB-S	Yes	13	0	46.2
Port Moresby	CRB-S, CRB-G	Yes (only CRB-S)	13	69.2	23.1
Philippines	***CRB-G***	***No***	***12***	***100***	***0***
Negros	CRB-G	No	12	100	0
Samoa	***CRB-S***	***Yes***	***31***	***0***	***64.5***
Upolo	CRB-S	Yes	25	0	72
Savai'i	CRB-S	Yes	6	0	33.3
Solomon Islands	***CRB-G***	***No***	***10***	***100***	***0***
Honiara	CRB-G	No	10	100	0

[a] Countries locations are indicated using in bold and italics. Where appropriate, specimen collection points within particular regions of a country are listed underneath.

[b] CRB collected from Johor and Terangganu (OrNV Type A), and from Perak (OrNV Type B), as defined in Ramle et al. (2005).

[c] *O. rhinoceros* first entered Palau in 1942 (Gressitt, 1953), with CRB-G likely to be a second invasion (ca 2000s) due to the recent reports of increased levels of severe damage. OrNV was detected in both CRB-S (3 of 3 from Aimeliik) and CRB-G (3 of 5 from Aimeliik, and 2 of 2 from Airai) in Palau.

[d] *O. rhinoceros* invaded the outer islands of PNG from 1942 to 1960 (Catley, 1969). CRB was first detected near Port Moresby (ca 2009), with both OrNV susceptible (CRB-S) and OrNV tolerant (CRB-G) haplotypes detected. OrNV was not detected in any CRB-G specimens from Port Moresby.

[e] The CRB-G haplotype designation was based on DNA analysis showing similarity to specimens identified from Guam, with CRB-S represent CRB specimens associated with known susceptibility to OrNV infection.

[f] Based on dilution PCR assays optimized to distinguish OrNV infection from gut tissue versus simple presence. ND, not determined as detection of OrNV infection based on tissue from legs has an unreliable association of OrNV presence.

[g] Individual specimens included were analyzed for both haplotype and virus detection.

Fig. 1 provides a representative example of the PCR-RFLP results observed. This PCR-RFLP technique offers a relatively quick, simple, and cheap molecular technique to distinguish the CRB-G populations from other CRB populations found in the Pacific.

To begin demarcating the range for CRB-G and identify potential source populations, the PCR-RFLP technique was used to survey several hundred CRB specimens obtained from areas harboring both established CRB populations and newly invaded sites. A library of 367 CRB population profiles was assembled using DNA extracted from CRB tissue specimens collected from 13 diverse geographic locations within the Asia-Pacific region (American Samoa, Diego Garcia, Fiji, Guam, Hawai'i, India, Indonesia, Malaysia, Palau, Papua New Guinea, Philippines, Samoa, and Solomon Islands). Table 2 summarizes these results and revealed that the CRB-G haplotype was present in Guam, Hawai'i, Indonesia, Palau, Papua New Guinea (Port Moresby region), Philippines, and Solomon Islands. All specimens obtained from Guam, Hawai'i, Philippines, and Solomon Islands shared the CRB-G PCR-RFLP pattern. Four of 7 specimens from Indonesia, 8 of 11 from Palau and 14 of 17 from Port Moresby were also found to be of the CRB-G haplotype. The remaining specimens from Indonesia, Palau, Port Moresby, and all other locations sampled displayed the more commonly observed CRB-OrNV susceptible PCR-RFLP haplotype pattern.

To gain preliminary insight into the relationships between the various CRB populations that were sampled, 152 CRB specimens were sequenced across the *COI* 'universal barcode' region, which encompasses the small region used in the PCR-RFLP haplotype assay. A subset of 31 sequences representative of each location was used to create a multiple alignment across a 676 bp region of the *COI* gene, which was followed by analyses of the variation observed at this locus among the CRB populations. From the unrooted tree construction shown in Fig. 2, four main groupings can be distinguished (clades I to IV). The CRB-G haplotype formed a distinct grouping separate from the other CRB haplotypes (clades II-IV). Within the CRB-G haplotype grouping (clade I) evidence for two further divisions were observed. Members of CRB-G^A subtype appeared to originate from a single original source population (100% sequence identity observed; seen as a group of eight sequences in Fig. 2) and are represented by populations identified from Guam, Hawai'i, Indonesia, Philippines, Port Moresby, Solomon Islands, plus a subset of specimens from Palau. The second cluster (CRB-G^B) was represented solely by a second subset of Palau specimens (seen as a group of two sequences in Fig. 2). This observation potentially represents a recombination event (Tsaousis et al., 2005) between the CRB-G^A group and a third subset population in Palau; putatively the original invasive CRB population into Palau that may be represented within clade IV. Clade IV also includes specimens obtained from Malaysia, India, and Indonesia. Alternatively, Palau may have been invaded by a second CRB-G-like population from a different source to the CRB-G^A subtype.

Of the two other main groupings, clades II and III correlated well with the reported historical pattern of CRB invasion into the Pacific (Catley, 1969). Clade II is composed of specimens collected from PNG and Malaysia. When the CRB-G specimens identified from Port Moresby were excluded, all remaining PNG specimens shared 100% sequence identity. Clade III contained members from Samoa, Fiji, Diego Garcia, India, and Malaysia. Specimens within Clade III displayed a 100% sequence conservation for all specimens except for one from Samoa (A35) and one from Malaysia (M1-10). The minor division within clade III involving the individual Malaysia and Samoa specimens (seen as two sequences in Fig. 2) may be due to a recent secondary introduction of a Malay CRB into Samoa. However, it is of more interest to point out that historically Sri Lanka was acknowledged as the original source of CRB for the 1909 Samoan introduction. From Samoa, CRB then spread into American Samoa, Fiji, and other surrounding Polynesian Islands (Catley, 1969). Here we provide genetic evidence supporting this claim, since it is widely held that CRB entered Sri Lanka from India. Records also showed that CRB invaded Diego Garcia from India during World War I via troop movements (Bedford, 1980; Orian, 1958). Clade IV nominally encompassed CRB specimens from India, Indonesia, Malaysia, and Palau. As opposed to the case in Polynesia, the origin of the CRB introductions into Palau and the outer islands of PNG (New Britain, New Ireland and Manus) were less clear, likely due to the

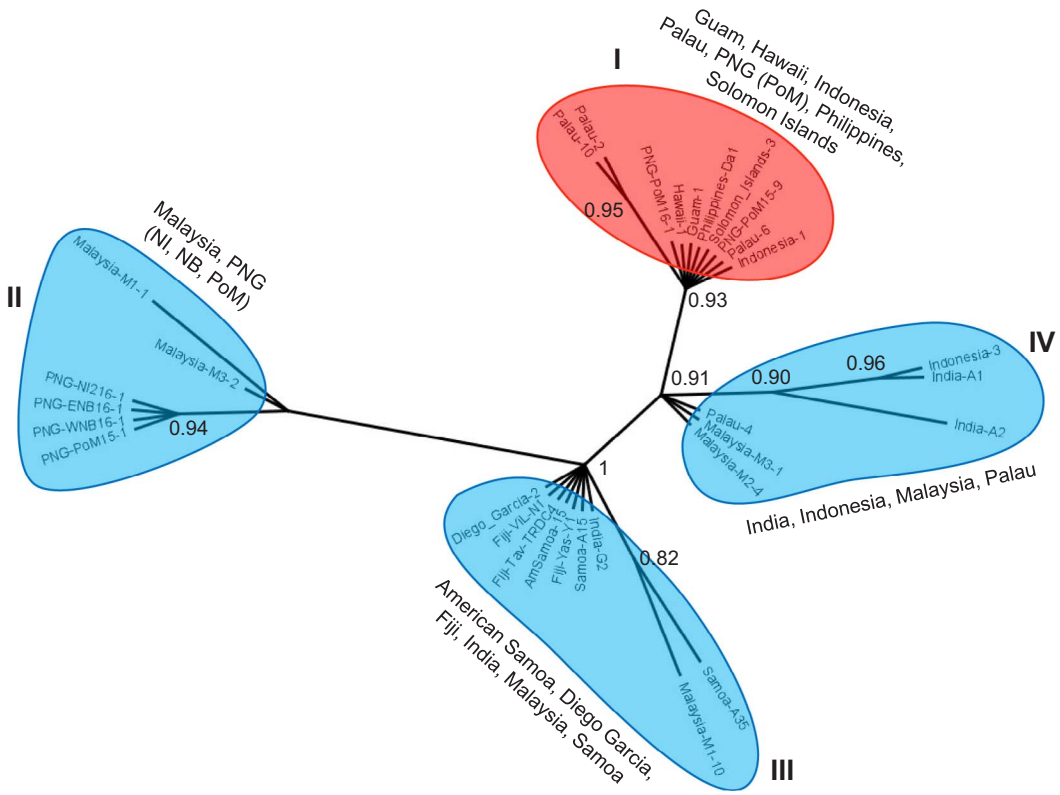

Fig. 2. Phylogeny of *O. rhinoceros* based on partial *COI* gene sequence. The unrooted majority rule consensus tree includes 31 representative CRB partial COI sequences (676 bp fragment between positions 1490–2198 (Folmer et al., 1994; Simon et al., 2006) from specimens obtained at various geographic locations (see Section 2.1.2 for associated for Genbank accession numbers). The constructed tree was inferred from Bayesian phylogenetic analysis as implemented in Geneious R8.0. Posterior probability values are shown at branch nodes. Individual clade groupings are labelled with roman numerals. Red shading (clade I) highlights the OrNV-tolerant CRB-G haplotype (based on DNA sequencing) with A and B subtypes also indicated, while the blue shading (clades II–IV) emphasizes CRB populations associated with susceptibility to OrNV infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chaotic nature of the wartime activities. The data summarized in Fig. 2 and Table 2 has suggested the possibility that accidental introduction of CRB into Micronesia and Melanesia arose from a location either within Malaysia or another nearby region.

With the center of origin for CRB believed to be located somewhere near the area bordering Continental and South East Asia (Bedford, 1976), an important component of this research was to begin identifying possible native sources for the invading OrNV-tolerant CRB-G haplotype. In addition to improving management of biosecurity risks, knowing the native range for CRB-G would provide an opportunity to search for candidate biocontrol agents able to deliver effective management of the invasive CRB-G biotype by reducing population number, which would reduce palm damage in infested areas, and prevent further spread into new regions. The data summarized in Fig. 2 suggests that the locations sampled from within Malaysia and India are unlikely to be candidate sources for CRB-G, which has assisted in excluding some regions from the search.

3.3. *O. rhinoceros* population-virus interactions

Concurrent with the CRB-G investigation, dilution PCR analysis of CRB tissue extracted DNA was used to detect the incidence of OrNV infection from 318 CRB specimens of confirmed haplotype. Results (see Table 2) revealed that 100% of the CRB-G specimens from the new outbreak areas of Guam, Hawai'i, Indonesia, Philippines, Port Moresby, and Solomon Islands, were negative for OrNV, indicating no detectable OrNV infection was present in these locations. Within the Palau specimens analyzed, five of the eight CRB-G specimens were positive for OrNV, while all three of the non-CRB-G haplotype specimens (i.e. non-CRB-G haplotype) were positive for OrNV infection. Interestingly, in Port Moresby both CRB-G and CRB-S were also found together; however, no OrNV was detected in CRB-G (nine specimens), while three of the four CRB-S collected were positive for OrNV. Moreover, no OrNV was detected in specimens from Indonesia or Philippines, which are both known to have widespread OrNV presence. From all of the other non-CRB-G regions sampled, where greater than five individuals were collected, OrNV infection was detected in a proportion of the CRB individuals ranging from 45.2% to 64.5% (Table 2).

Fig. 3 presents a map summarizing the current known distribution for CRB-G populations in the Asia-Pacific region and reveals that most locations with confirmed CRB-G populations are generally not infected by OrNV, i.e. Guam, Hawai'i, Port Moresby, Solomon Islands, Indonesia, and Philippines. Furthermore, this absence of OrNV infection in CRB-G was correlated with severe to lethal levels of palm damage being reported in these areas (see Supplemental Fig. S1 for exemplar photos). OrNV infection is known to be associated with all other CRB populations (Table 2, Fig. 3), and with palm damage being reported as light to moderate in these areas (data not shown). Palau appears to be the exception to the observation that CRB-G beetles are uninfected by OrNV, although the PCR positive results could be due to cross contamination. Further validation will be required to confirm these observations, but it is notable that Palau has also reported increased palm damage since 2010, and the severity of palm damage has significantly increased over time. In other areas where CRB-G was found to cohabitate with other (OrNV susceptible) CRB populations (e.g. Port Moresby), or where OrNV presence has been historically widespread (e.g. Indonesia, Philippines) (Hajek et al., 2007; Jackson, 2009), OrNV was not detected from the CRB-G specimens.

The results defining the pattern of CRB-G distribution together with observations of scarce OrNV infection in CRB-G (even when OrNV is

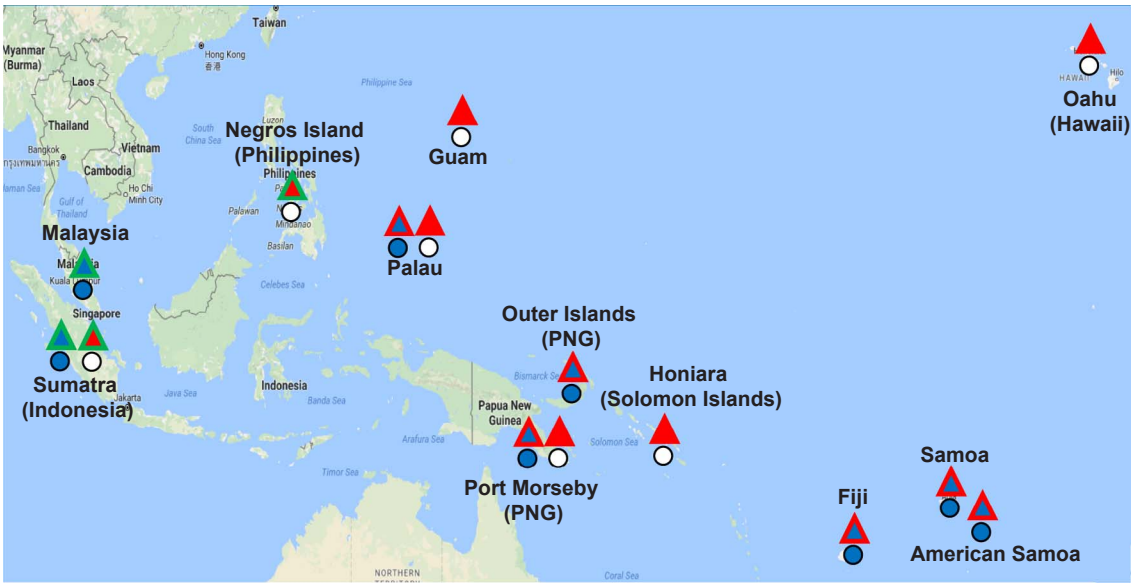

Fig. 3. Distribution of *O. rhinoceros* and *Oryctes rhinoceros* nudivirus from specimen collections in the South East Asia-Pacific region. Triangles indicate reported presence of *O. rhinoceros* in a location whereby a green outline indicates native CRB range, a red outline signifies CRB invaded area, red shading indicates CRB-G present, and blue shading indicates non-CRB-G populations. Blue filled circles represent OrNV infection detected in CRB specimens, while white filled circles indicate no OrNV infection was detected. Note that, except for Palau, OrNV was not detected in any CRB-G specimens, even when virus was known to be present within region. The background map is a screen shot from Google Maps (MapData 2016, www.google.co.nz/maps). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

present in the area) and correlated high levels of palm damage in the outbreak areas, are suggestive of OrNV tolerance in CRB-G populations as they are not being incapacitated by infection from virus currently used to manage CRB in the Pacific. The widespread heavy damage to palm trees caused by CRB-G (see Fig. S1) is reminiscent of observations of CRB impact in the Pacific prior to the release of OrNV in the biological control campaigns of the 1960s and 70s (Huger, 2005). The re-emergence of the coconut rhinoceros beetle problem with severe damage associated with a virus-free beetle also provides validation of the long-term impact of the original OrNV releases where virus has been maintained in the treated populations and damage has remained low (Bedford, 2013b; Huger, 2005). Interestingly in relation to resistance, Zelazny et al. (1989) reported lower than expected mortality when CRB collected from the Philippines were challenged with OrNV isolates known to infect CRB collected from Samoa. From these observations they postulated that an OrNV resistant population of CRB may be present within the Philippines. The current data presented here appears to corroborate their hypothesis as the CRB-G haplotype was orally tolerant to infection by several OrNV isolates, and potentially the dominant (if not sole) population within a localized area of the Philippines. Observations within this area did not show signs of severe CRB palm damage as is seen in other CRB-G infested regions (unpublished data).

Irrespective of the specific mechanism limiting infection of CRB-G by the OrNV isolates tested so far, future research is essential to identify effective biocontrol agents to assist with management of CRB-G. Of relevance with respect to improving CRB-G population control within invaded Pacific regions was the identification of CRB-G specimens from Indonesia and the Philippines, which are both considered native locations for CRB. Aside from CRB palm damage resulting from felled vegetation due to recent cyclones (Philippines) or development of new oil palm plantations (Indonesia), severe CRB damage has not been recently reported. Native habitat provides a good opportunity to identify candidate pathogens or other organisms for use as effective CRB-G biocontrol agents; this approach was previously successful in achieving CRB population control from the original series of CRB invasions (Huger, 2005). Although the OrNV isolates tested here did not orally infect CRB-G, considerable genetic variation has been documented among OrNV isolates, and research within new island release areas have shown rapid evolution of the virus (Crawford et al., 1985, 1986; Crawford and Zelazny, 1990). This suggests there is a good chance to identify an effective OrNV isolate or other control option. Recognition of the CRB-G haplotype having escaped control from the commonly used OrNV isolates has highlighted the importance of actively overseeing and maintaining management programmes for important established insect pests, even when it appears a robust solution has been found.

4. Conclusion

The CRB-G haplotype identified here is genetically distinct from other CRB populations already established in the Pacific region and is highly damaging to palms. The evidence provided demonstrates CRB-G is not appreciably affected by the OrNV isolates commonly used for biocontrol management of other CRB populations. Conversely, identification of the CRB-G haplotype has highlighted how effective and important the use of the OrNV biocontrol agent was for effective management of the other CRB populations that invaded the Pacific region. Further invasion and spread of CRB-G poses a serious threat to the Pacific islands and states, particularly through its potential to damage and kill the culturally iconic and economically vital palm trees. Over the past decade, CRB-G has spread to uninfested islands at a rate of about one new island every two years. This is especially worrying for atolls and small islands, where coconut is an essential source of food and building material. However, the current findings have identified the cause of the problem and indicate that new biocontrol agents/strains will be needed. Furthermore, we have identified candidate locations to begin the search for potential biocontrol agents to assist with establishing effective CRB-G population control. This information also provides an important base for developing future biosecurity policies and improving CRB management efforts for the Pacific region and beyond.

Acknowledgments

We thank Dr. Russell Campbell (Guam Plant Inspection Facility) for generously allowing use of lab space to carry out CRB-OrNV bioassays, Natalia J. Vandenburg at the USDA Systematic Entomology Laboratory

(Beltsville, MD, USA) for providing morphological species identification, Mr. Roland Quitugua (University of Guam) for assistance with CRB collection and discussions. We also thank the following people for their invaluable assistance with collection of CRB specimens: Mr. Solomon Sar and Dr. Mark Eros (Papua New Guinea Oil Palm Research Association); Mr. Apenisa Sailo and Mrs. Aradhana Deesh (Fiji Ministry of Primary Industries); Mr. Billy Enosa (Samoa Ministry of Agriculture and Fisheries); Dr. Ramle Moslim (Malaysian Palm Oil Board); Dr. Charlotte Pushpunjara (University of Canterbury, New Zealand). Thank you to Gribbles Veterinary Services (Christchurch, New Zealand) for assistance with preparing histology slides. Funding for this research was provided through the New Zealand Ministry of Foreign Affairs and Trade (contract CON/517/4SSDPF), and several grants to the University of Guam from USDA-APHIS and the USDA Forest Service.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2017.07.006.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Bedford, G.O., 1976. Use of a virus against the coconut palm rhinoceros beetle in Fiji. Pest Articles News Summar. 22, 11–25.

Bedford, G.O., 1980. Biology, ecology, and control of palm rhinoceros beetles. Annu. Rev. Entomol. 25, 309–339.

Bedford, G.O., 2013a. Biology and management of palm dynastid beetles: recent advances. Annu. Rev. Entomol. 58, 353–372.

Bedford, G.O., 2013b. Long-term reduction in damage by rhinoceros beetle *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae: Dynastinae) to coconut palms at *Oryctes* Nudivirus release sites on Viti Levu, Fiji. Afr. J. Agricult. Res. 8, 6422–6425.

Berringer, D., 2007. Coconut Rhinoceros Beetle Eradication Program: Guam. USDA Environmental Assessment (GU-08-1).

Catley, A., 1969. The Coconut Rhinoceros Beetle *Oryctes rhinoceros* (L)[Coleoptera: Scarabaeidae: Dynastinae]. Pest Articles News Summar. 15, 18–30.

Crawford, A.M., 1982. A coleopteran cell line derived from *Heteronychus arator* (Coleoptera: Scarabaeidae). In Vitro 18, 813–816.

Crawford, A.M., Ashbridge, K., Sheehan, C., Faulkner, P., 1985. A physical map of the *Oryctes* baculovirus genome. J. Gen. Virol. 66, 2649–2658.

Crawford, A.M., Sheehan, C., 1984. An *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae) baculovirus inoculum derived from tissue culture. J. Econ. Entomol. 77, 1610–1611.

Crawford, A.M., Zelazny, B., 1990. Evolution in *Oryctes* baculovirus: rate and types of genomic change. Virology 174, 294–298.

Crawford, A.M., Zelazny, B., Alfiler, A.R., 1986. Genotypic variation in geographical isolates of *Oryctes* baculovirus. J. Gen. Virol. 67, 949–952.

Endrödi, S., 1985. The Dynastinae of the World. Springer, The Netherlands.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotech. 3, 294–299.

Gressitt, J.L., 1953. The Coconut Rhinoceros Beetle (*Oryctes rhinoceros*) with particular reference to the Palau Islands. Bernice P. Bishop Museum Bull. 212, 1–157.

Hajek, A.E., McManus, M.L., Delalibera Júnior, I., 2007. A review of introductions of pathogens and nematodes for classical biological control of insects and mites. Biol. Control 41, 1–13.

Hebert, P.D.N., Cywinska, A., Ball, S.L., DeWaard, J.R., 2003a. Biological identifications through DNA barcodes. Proc. R. Soc. B: Biol. Sci. 270, 313–321.

Hebert, P.D.N., Ratnasingham, S., DeWaard, J.R., 2003b. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. B: Biol. Sci. 270, S96–S99.

Huger, A.M., 1966. A virus disease of the Indian rhinoceros beetle, *Oryctes rhinoceros* (linnaeus), caused by a new type of insect virus, *Rhabdionvirus oryctes* gen. n., sp. n. J. Invertebr. Pathol. 8, 38–51.

Huger, A.M., 2005. The *Oryctes* virus: Its detection, identification, and implementation in biological control of the coconut palm rhinoceros beetle, *Oryctes rhinoceros* (Coleoptera: Scarabaeidae). J. Invertebr. Pathol. 89, 78–84.

Jackson, T.A., 2009. The use of *Oryctes* virus for control of rhinoceros beetle in the Pacific Islands. In: Hajek, A.E., Glare, T., O'Callaghan, M. (Eds.), Use of Microbes for Control and Eradication of Invasive Arthropods. Springer, Dordrecht, The Netherlands, pp. 113–140.

Jacob, T., 1996. Introduction and establishment of baculovirus for the control of rhinoceros beetle *Oryctes rhinoceros* (Coleoptera: Scarabaeidae) in the Andaman Islands (India). Bull. Entomol. Res. 86, 257–262.

Jayawardena, S.P., 2013. Effective inoculation method and optimum concentration of *Oryctes* virus for biological control of coconut beetle (*Oryctes rhinoceros*) adults. Euro. Int. J. Sci. Technol. 2, 188–194.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649.

Khudri, N.A.F.R.S., Azmi, W.A., Kamarudin, N., Ali, S.R.A., Moslim, R., 2016. Replication of *Oryctes* nudivirus (OrNV) in insect cell line DSIR-HA-1179 and its infectivity on neonates of rhinoceros beetle, *Oryctes rhinoceros*. J. Oil Palm Res. 28, 452–462.

Kiernan, J.A., 1990. Histological and Histochemical Methods: Theory and Practice. Pergamon Press, Toronto.

Lacey, L.A., 2012. Manual of Techniques in Invertebrate Pathology, second ed. Academic Press, San Diego.

Meyer, C.P., Paulay, G., 2005. DNA barcoding: error rates based on comprehensive sampling. PLoS Biol. 3, e422.

O'Leary, N.A., Wright, M.W., Brister, J.R., Ciufo, S., Haddad, D., McVeigh, R., Rajput, B., Robbertse, B., Smith-White, B., Ako-Adjei, D., Astashyn, A., Badretdin, A., Bao, Y., Blinkova, O., Brover, V., Chetvernin, V., Choi, J., Cox, E., Ermolaeva, O., Farrell, C.M., Goldfarb, T., Gupta, T., Haft, D., Hatcher, E., Hlavina, W., Joardar, V.S., Kodali, V.K., Li, W., Maglott, D., Masterson, P., McGarvey, K.M., Murphy, M.R., O'Neill, K., Pujar, S., Rangwala, S.H., Rausch, D., Riddick, L.D., Schoch, C., Shkeda, A., Storz, S.S., Sun, H., Thibaud-Nissen, F., Tolstoy, I., Tully, R.E., Vatsan, A.R., Wallin, C., Webb, D., Wu, W., Landrum, M.J., Kimchi, A., Tatusova, T., DiCuccio, M., Kitts, P., Murphy, T.D., Pruitt, K.D., 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 44, D733–D745.

Orian, A.J.E., 1958. Report on a visit to Diego Garcia. La Revue Agricole et Sucrière de l'Ile Maurice. 38, 127–143.

Pushparajan, C., Marshall, S.D.G., Claus, J.D., Visnovsky, G., 2013. Characterization of growth and *Oryctes* virus production in attached cultures of the DSIR-HA-1179 coleopteran insect cell line. Cytotechnology 65, 1003–1016.

R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria < https://www.R-project.org/ > .

Ramle, M., Wahid, M.B., Norman, K., Glare, T.R., Jackson, T.A., 2005. The incidence and use of *Oryctes* virus for control of rhinoceros beetle in oil palm plantations in Malaysia. J. Invertebr. Pathol. 89, 85–90.

Richards, N.K., Glare, T.R., Aloali'i, I., Jackson, T.A., 1999. Primers for the detection of *Oryctes* virus from Scarabaeidae (Coleoptera). Mol. Ecol. 8, 1552–1553.

Secretariat of the Pacific Community, 2015. An emerging biotype of Coconut Rhinoceros Beetle Discovered in the Pacific. Pest Alert. 51.

Simon, C., Buckley, T.R., Frati, F., Stewart, J.B., Beckenbach, A.T., 2006. Incorporating molecular evolution into phylogenetic analysis, and a new compilation of conserved polymerase chain reaction primers for animal mitochondrial DNA. Annu. Rev. Ecol. Evol. Syst. 37, 545–579.

Tsaousis, A.D., Martin, D.P., Ladoukakis, E.D., Posada, D., Zouros, E., 2005. Widespread recombination in published animal mtDNA sequences. Mol. Biol. Evol. 22, 925–933.

Zelazny, B., 1973. Studies on *Rhabdionvirus oryctes*. II. Effect on adults of *Oryctes rhinoceros*. J. Invertebr. Pathol. 22, 122–126.

Zelazny, B., 1976. Transmission of a baculovirus in populations of *Oryctes rhinoceros*. J. Invertebr. Pathol. 27, 221–227.

Zelazny, B., 1978. Methods of inoculating and diagnosing the baculovirus disease of *Oryctes rhinoceros*. FAO Plant Prot. Bull. 26, 163–168.

Zelazny, B., 1979. Virulence of the baculovirus of *Oryctes rhinoceros* from ten locations in the Philippines and in Western Samoa. J. Invertebr. Pathol. 33, 106–107.

Zelazny, B., Alfiler, A.R., Lolong, A., 1989. Possibility of resistance to a baculovirus in populations of the coconut rhinoceros beetle (*Oryctes rhinoceros*). FAO Plant Prot. Bull. 37, 77–82.