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Coconut Rhinoceros Beetle Biological Control

Aubrey Moore, University of Guam

February 23, 2018

Contents

1 Project Initiation

- Project and finances were established under the Research Corporation of the University of Guam.

2 Staffing

- Ian Iriarte, my graduate student who worked on this project resigned so that he could work with the Guam Department of Agriculture on another invasive species, the little fire ant.

- A grant proposal to hire a post-doc entomologist to work specifically on CRB biocontrol was funded by the Department of Interior, Office of Island Affairs. A job description and position announcement was written (Appendix C: Post-doctoral Entomologist Position Announcement) and posted on the University of Guam web site and on the Entomological Society of America web site. Additional technical assistance will be recruited once the post-doc is on-board.

3 Research Progress

- Permit applications to allow importation of *Oryctes rhinoceros* nudivirus (OrNV) isolates were submitted to USDA-APHIS and the Guam Department of Agriculture. These were approved. The first isolate to be imported under these permits was collected from CRB Guam biotype adult beetle caught in the Dumaguete area of Negros Island, Philippines during field work conducted there by Dr. Aubrey Moore (UOG), Ian Iriarte (UOG), and Dr. Sean Marshall (AgResearch, New Zealand). The isolate was cleaned up and propagated at AgResearch New Zealand prior to being sent to Guam for pathogenicity testing.

- Field collected CRB-G adults which were challenged with a high dose of OrNV showed no significant response (Appendix B: Technical Report: Bioassay of OrNV Isolated from CRB-G, Negros Island, Philippines).

- An environmental cabinet which will allow control of temperature, relative humidity, and lighting during bioassays was procured and installed.

4 Publication

- A refereed journal article entitled *A new haplotype of the coconut rhinoceros beetle, Oryctes rhinoceros, has escaped biological control by Oryctes rhinoceros nudivirus and is invading Pacific Islands* was published in the Journal of Invertebrate Pathology (Appendix A: Publication).

- I published an interactive, online map of the geographical distribution of CRB and CRB-G at http://aubreymoore.github.io/crbdist/mymap.html.

5 Appendix A: Publication

See following page.

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

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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

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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

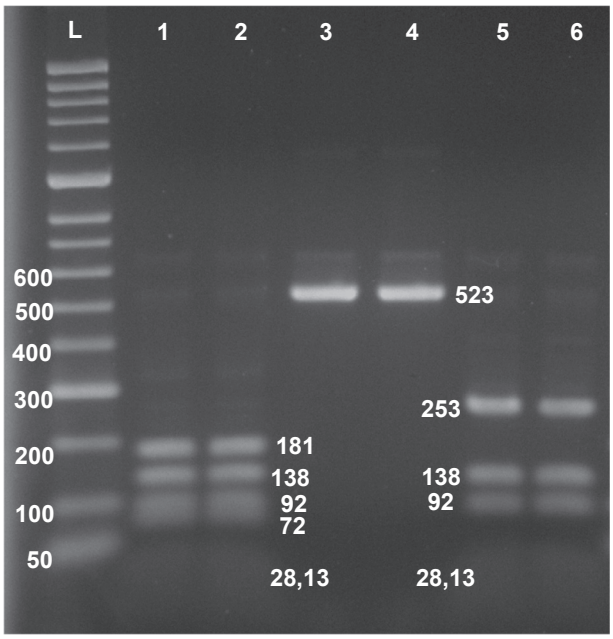

Fig. 1. Exemplar PCR-RFLP analysis results that distinguish the CRB-G haplotype from other CRB populations. Results shown are examples of CRB-S (lanes 1–3) and CRB-G (lanes 4–6) haplotype specimens. Following PCR amplification of a 523 bp fragment of the *COI* gene from *O. rhinoceros* DNA, the amplicons 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

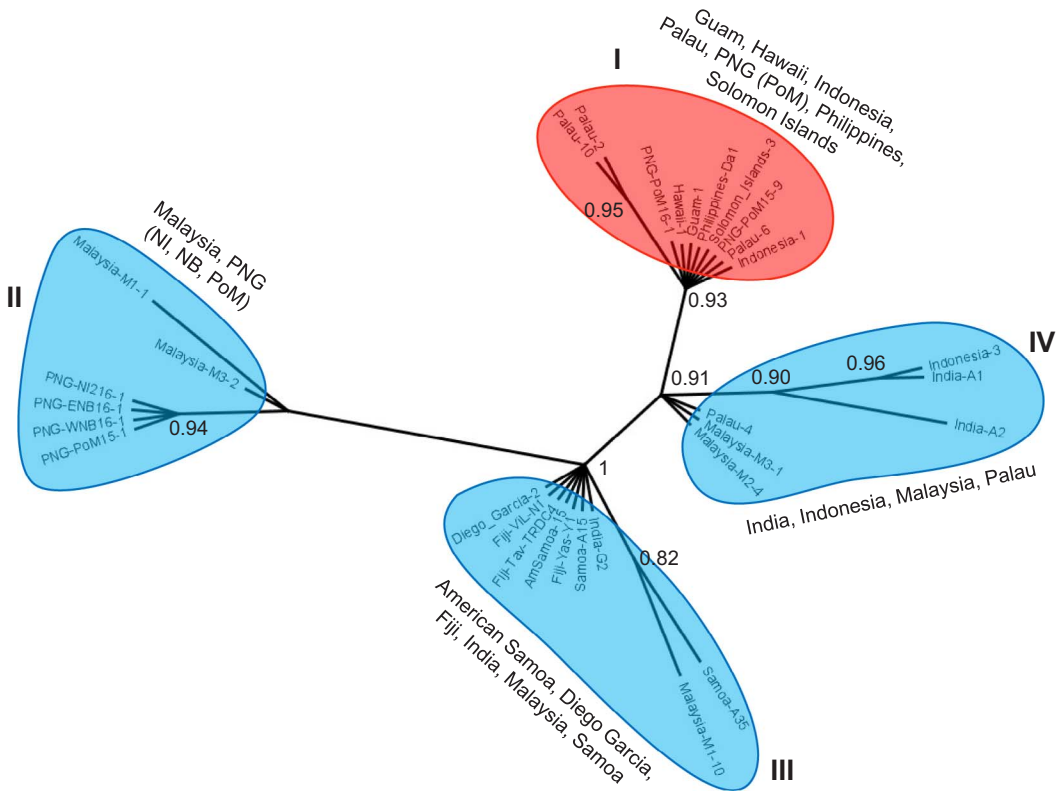

Fig. 2. Phylogeny of *O. rhinoceros* based on partial *COI* gene sequence. The unrooted majority rule consensus tree includes 31 representative CRB partial COI sequences (676 bp fragment between positions 1490–2198 (Folmer et al., 1994; Simon et al., 2006) from specimens obtained at various geographic 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

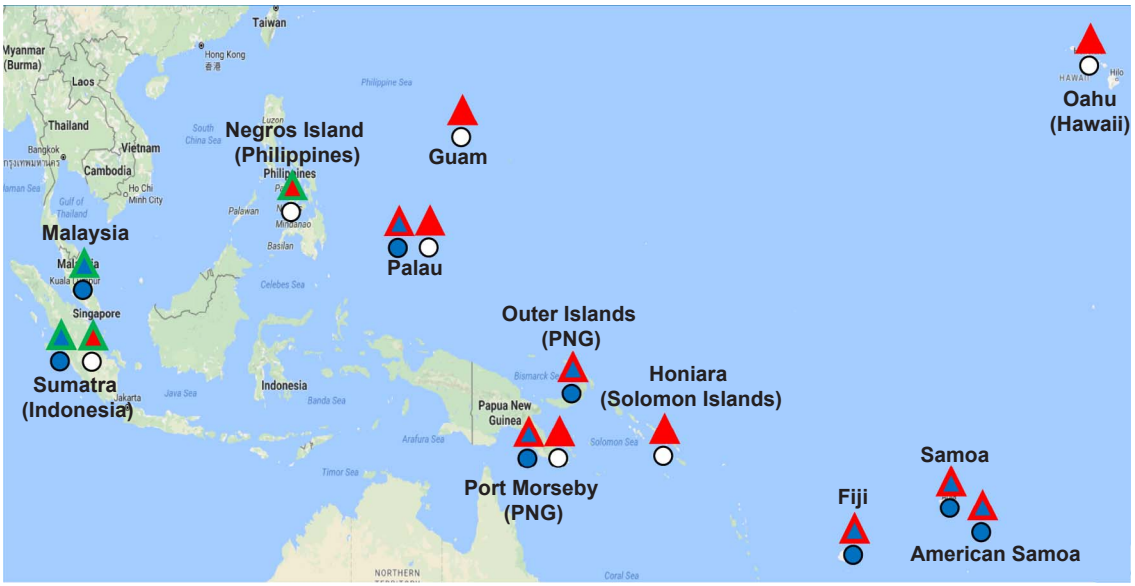

Fig. 3. Distribution of *O. rhinoceros* and *Oryctes rhinoceros* nudivirus from specimen collections in the South East Asia-Pacific region. Triangles indicate reported presence of *O. rhinoceros* in a location whereby a green outline indicates native CRB range, a red outline signifies CRB invaded area, 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.

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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.

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6 Appendix B: Technical Report: Bioassay of OrNV Isolated from CRB-G, Negros Island, Philippines

See following page.

Dumaguete OrNV bioassay 1

January 11, 2018

1 Dumaguete OrNV Isolate - Bioassay 1

1.1 Introduction

This notebook documents an initial bioassay of the Dumaguete isolate of *Oryctes* nudivirus.

1.2 Materials and Methods

1.2.1 Virus Sample

This virus was isolated from a single infected *Oryctes rhinoceros* Guam biotype specimen collected near Dumaguete, Negros Island, Philippines in January 2017.

1.2.2 Test Insects

Test insects were collected weekly from 31 coconut rhinoceros beetle pheromone traps (barrel traps) at the University of Guam Agricultural Experiment Station at Yigo. Beetles were held individually in numbered Mason jars partially filled with moist peat moss which were stored in an environmental cabinet at a temperature of 80 degrees F. Each was fed a slice of banana weekly. Beetles were reared an average of 46 days (range: 23 d to 75 d) prior to treatment.

1.2.3 Treatment

- 20 beetles were selected at random for treatment and another 20 were selected for experimental control
- 100 mg of sucrose was disolved in a 1 ml sample of the virus. Each beetle was given a 40 microlitre dose of this solution by pipetting a drop onto its mouthparts. A placebo was not given to beetles in the experimental control group.
- All beetles were provided a slice of banana on the day following treatment.

1.2.4 Observation procedure

- Beetles were observed weekly.
- Each beetle was weighed and its mass was entered immediately into a spreadsheet.
- A slice of banana was added immediately after each beetle was returned to its jar.

1.2.5 Analysis

- The data model includes 2 tables saved as Excel spreadsheets (see below).
- An empty **mass** field in the **observations** table indicates that the beetle died prior to observation.

1.3 Results and Discussion

- The beetles did not readily drink the 40 microlitre dose applied to their mouthparts. A food incorporation bioassay may be more efficient.
- During the 28 day bioassay period, 1 of 20 virus treated beetles died and 4 of 20 beetles in the experimental control group died. The difference in mortality is not significant (p = 0.34; Fisher's exact test). Post mortems indicated that all of the dead beetles were infected with *Metarhizium majus*. Guts looked normal.
- There was a significant difference in weight loss during the first week of the bioassay (p = 0.02; Welch's t-test). However, there was no significant difference in weight loss during succeding weeks. Difference in weight loss during the first week may be due to the fact that the virus treated beetles were handled much more than the contol group.
- In conclusion, results from this initial bioassay does not indicate pathogenicity to CRB-G for the OrNV Dumaguete isolate.

1.4 Calculations

```
In [22]: import pandas as pd
         import matplotlib.pyplot as plt
         import sqlite3
         import numpy as np
         from scipy.stats import ttest_ind, fisher_exact

         %matplotlib inline
         pd.options.display.max_rows = 4
```

1.4.1 import data from spreadsheet

```
In [23]: DATAFILE = 'aubrey.xlsx'

         df_jars = pd.read_excel(DATAFILE, 'jars')
         df_jars
```

```
Out[23]:     jar_number date_collected     sex treatment
         0           32     2017-10-02  Female     virus
         1           85     2017-10-02  Female     virus
         ..         ...            ...     ...       ...
         38        1263     2017-10-18    Male   control
         39        1633     2017-10-23    Male   control

         [40 rows x 4 columns]
```

```
In [24]: df_observations = pd.read_excel(DATAFILE, 'observations')
         df_observations
```

Out[24]:

	jar_number	observation_date	mass	note
0	32	2017-11-15	4487.0	NaN
1	50	2017-11-15	4136.0	mites
..	...	...	...	...
198	1263	2017-12-13	3036.0	NaN
199	1633	2017-12-13	3399.0	NaN

[200 rows x 4 columns]

In [25]:
```
# Create a dict containing pairs of dates.
# The index is an observation date and the value is the previous observation date

obs_dates = df_observations.observation_date.unique()
prev_obs_date = dict(zip(obs_dates[1:], obs_dates))

# Example usage:
# y = df_observations.observation_date[100]
# print y
# prev_obs_date[y.to_datetime64()]
```

In [26]:
```
# Add a new column "days_post_treatment" to "df_observations" and populate it

treatment_date = df_observations.observation_date.min().to_datetime64() # Assumes all t
df_observations['days_post_treatment'] = np.nan

for index, row in df_observations.iterrows():
    try:
        date = row.observation_date.to_datetime64()
        if date == treatment_date:
            days_post_treatment = 0
        else:
            days_post_treatment = date - treatment_date
            # Convert from timedelta in nanoseconds to integer days
            days_post_treatment = (days_post_treatment / np.timedelta64(1, 'D')).astype
    except:
        continue
    df_observations.loc[index, 'days_post_treatment'] = days_post_treatment

# Add a new column "mass_change" to "df_observations" and populate it

df_observations['mass_change'] = np.nan

for index, row in df_observations.iterrows():
    try:
        date = row.observation_date.to_datetime64()
        prev_date = prev_obs_date[date]
        prev_mass = int(df_observations.loc[(df_observations.observation_date==prev_dat
            (df_observations.jar_number==row.jar_number), 'mass'])
```

```
            mass_change = int(row.mass) - prev_mass
        except:
            continue
        df_observations.loc[index, 'mass_change'] = mass_change

df_observations
```

Out[26]:

	jar_number	observation_date	mass	note	days_post_treatment	mass_change
0	32	2017-11-15	4487.0	NaN	0.0	NaN
1	50	2017-11-15	4136.0	mites	0.0	NaN
..	...	...	...	...	...	...
198	1263	2017-12-13	3036.0	NaN	28.0	258.0
199	1633	2017-12-13	3399.0	NaN	28.0	-194.0

[200 rows x 6 columns]

In [27]:
```
df = df_observations.merge(df_jars, on='jar_number')
df
```

Out[27]:

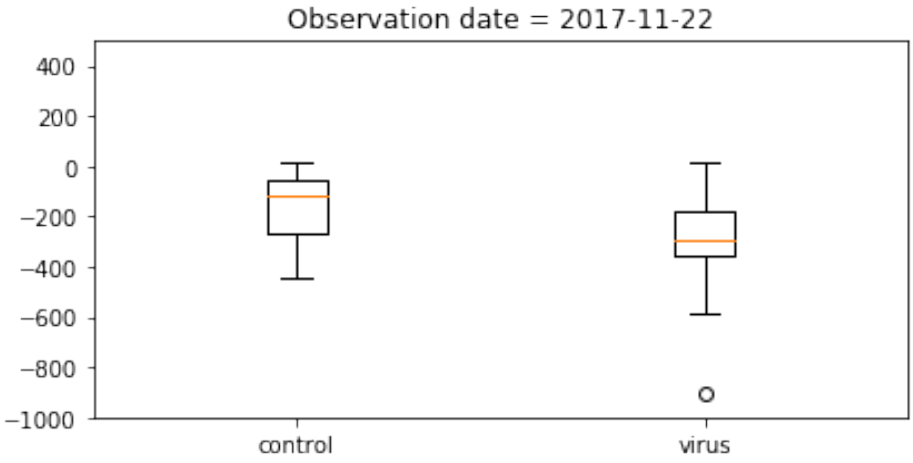
	jar_number	observation_date	mass	note	days_post_treatment	mass_change	date_collected	sex	treatment
0	32	2017-11-15	4487.0	NaN	0.0	NaN	2017-10-02	Female	virus
1	32	2017-11-22	3900.0	NaN	7.0	-587.0	2017-10-02	Female	virus
..	...	...	...	...	...	...	...	...	...
198	1633	2017-12-06	3593.0	NaN	21.0	447.0	2017-10-23	Male	control
199	1633	2017-12-13	3399.0	NaN	28.0	-194.0	2017-10-23	Male	control

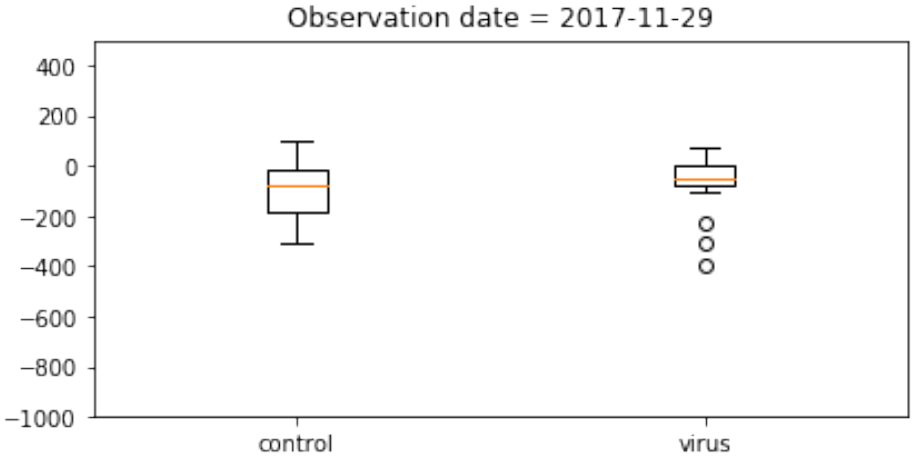
[200 rows x 9 columns]

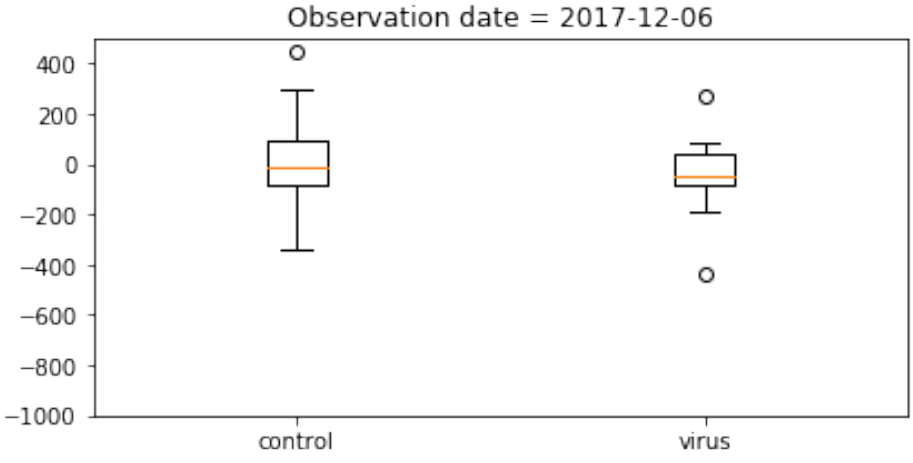
In [28]:
```
fig = plt.figure(figsize=(6, 12))
i = 0
print 'observation \tcontrol \t\tvirus'
print 'date \t\tmean mass change (mg) \tmean mass change (mg) \tp-value'
for date in prev_obs_date.keys():
    i += 1
    v = df.loc[(df.observation_date==date) &
```

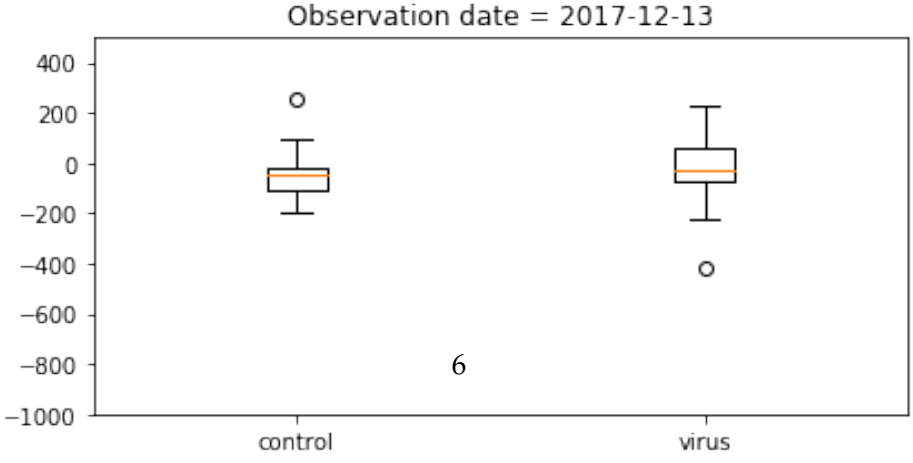
```
                                (df.treatment=='virus'), 'mass_change']
            v = v[-np.isnan(v)]
            c = df.loc[(df.observation_date==date) &
                                (df.treatment=='control'), 'mass_change']
            c = c[-np.isnan(c)]

            statistic, pvalue = ttest_ind(c, v, nan_policy='omit', equal_var=False)
            print '{}\t{:.0f} (n={})\t\t{:.0f} (n={})\t\t{:.4f}'.format(
                str(date)[:10], np.mean(c), len(c), np.mean(v), len(v), pvalue)
            ax = fig.add_subplot(4,1,i)
            title = 'Observation date = ' + str(date)[:10]
            ax.set_title(title)
            ax.boxplot([c.values, v.values])
            ax.set_xticklabels(['control', 'virus'])
            ax.set_ylim([-1000, 500])
        plt.tight_layout()
```

```
observation         control                 virus
date                 mean mass change (mg)         mean mass change (mg)         p-value
2017-11-22        -163 (n=20)                -298 (n=20)                0.0195
2017-11-29        -107 (n=19)                -70 (n=20)                0.3216
2017-12-06        3 (n=18)                -40 (n=20)                0.4429
2017-12-13        -41 (n=16)                -37 (n=19)                0.9350
```


Observation date = 2017-11-22
400
200
0
−200
−400
−600
−800
−1000
control
virus


Observation date = 2017-11-29
400
200
0
−200
−400
−600
−800
−1000
control
virus


Observation date = 2017-12-06
400
200
0
−200
−400
−600
−800
−1000
control
virus

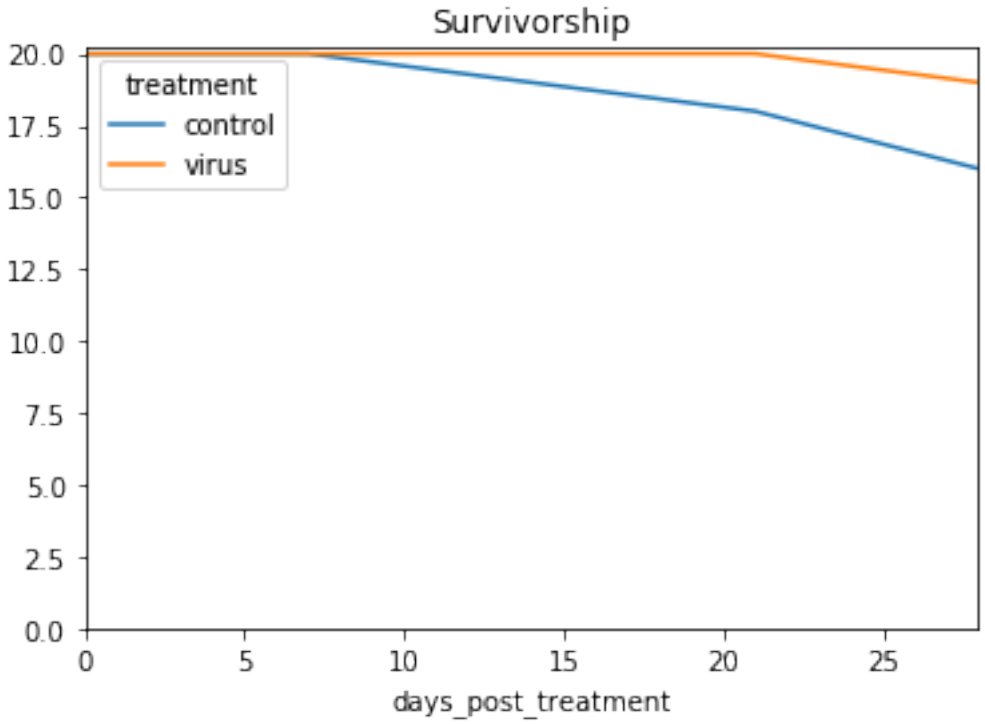

Observation date = 2017-12-13
400
200
0
−200
−400
−600
−800
−1000
control
virus

1.4.2 Mortality

In [29]:
```
df_groupby = df.groupby(['days_post_treatment','treatment'])['mass'].count().unstack()
df_groupby
```

Out[29]:
```
treatment             control  virus
days_post_treatment
0.0                        20     20
7.0                        20     20
...                       ...    ...
21.0                       18     20
28.0                       16     19

[5 rows x 2 columns]
```

In [34]:
```
myplot = df_groupby.plot()
ylim = myplot.axes.get_ylim()
myplot.axes.set_ylim(0, ylim[1])
myplot.set_title('Survivorship');
```



In [31]:
```
c_total = df_groupby.control.max()
c_alive = df_groupby.control.min()
```

```
        c_dead = c_total - c_alive
        v_total = df_groupby.virus.max()
        v_alive = df_groupby.virus.min()
        v_dead = v_total - v_alive
        print '{}\t{}\t{}'.format('', 'alive', 'dead')
        print '{}\t{}\t{}'.format('control', c_alive, c_dead)
        print '{}\t{}\t{}'.format('virus', v_alive, v_dead)
        print
        oddsratio, pvalue = fisher_exact([[c_alive, c_dead], [v_alive, v_dead]])
        print 'Fisher''s exact test p-value = {}'.format(pvalue)
```

```
        alive   dead
control 16      4
virus   19      1

Fishers exact test p-value = 0.341649341649
```

1.4.3 Holding time prior to treatment

```
In [32]: # Calculate days held prior to treatment

        pd.options.display.max_rows=100
        df_temp = df[df.observation_date==treatment_date]
        (df_temp.observation_date - df_temp.date_collected).describe()
```

```
Out[32]: count                         40
         mean            46 days 15:00:00
         std      18 days 08:07:05.397423
         min             23 days 00:00:00
         25%             33 days 00:00:00
         50%             44 days 00:00:00
         75%             57 days 00:00:00
         max             75 days 00:00:00
         dtype: object
```

7 Appendix C: Post-doctoral Entomologist Position Announcement

See following page.

RESEARCH CORPORATION
OF THE UNIVERSITY OF GUAM

The Research Corporation of the University of Guam does not discriminate on the basis of sex, race, color, religion, national or ethnic origin, disability unrelated to job requirements, age (except as permitted by law), citizenship status, marital status, or political affiliation. Furthermore, the Research Corporation of the University of Guam does not discriminate on the basis of sex in the admission to or employment in its educational programs or activities.

ANNOUNCEMENT

THE RESEARCH CORPORATION OF THE UNIVERSITY OF GUAM SOLICITS APPLICATIONS TO ESTABLISH A LIST OF ELIGIBLES FOR THE FOLLOWING LIMITED TERM APPOINTMENT, 100% FEDERALLY FUNDED FULL-TIME POSITION (SUBJECT TO THE AVAILABILITY OF FUNDS):

Position Title
Post-Doctoral Researcher (Insect Pathologist) **JOB # RC-18-06**

***Application Deadline:* Until position is filled**
Send RCUOG application, transcripts, curriculum vitae, and one-page personal statement that outlines pertinent experience, general qualifications, graduate degrees and research fields to rcuoghr@triton.uog.edu. The RCUOG application can be downloaded from www.uog.edu/rcuog; located under the Forms subhead.

Salary
Grade O, Step1, $23.99 per hour
Full-time, 40 hours per week
Benefits: Medical and Dental Insurance, 4 hours annual/4 hours sick leave per pay period, holiday pay, Social Security and Medicare

The position begins upon **Selection Notification** ends on **September 30, 2019** based on availability of funds with possibility of extension should additional funds become available.

Location:
College of Natural and Applied Sciences at the University of Guam, Mangilao, Guam

MINIMUM QUALIFICATIONS:

- Ph.D. from an accredited college or university in Entomology, Zoology, or a related field
- Must be a U.S. citizen or a permanent resident.

MINIMUM KNOWLEDGE, ABILITIES, AND SKILLS:

- Experience in insect pathology and insect rearing

- Experience in performing bioassays to evaluate insect pathogens as potential biological control agents
- Ability to conduct field work under sometimes difficult field conditions (e.g. high temperature and humidity)
- Knowledge of the principals and practice of biological control.
- Ability to create and implement project design
- Ability to collect, organize, and analyze data
- Experience in report writing
- Supervisory experience
- Must possess a valid driver's license and a valid passport.

CHARACTER OF DUTIES:

The post-doctoral researcher will work with a project PI and collaborators to develop effective biological control for the coconut rhinoceros beetle Guam biotype (CRB-G). This recently discovered biotype has recently invaded several Pacific island groups were it is causing damage and mortality of coconut and oil palms. In the past, coconut rhinoceros beetle invasions were successfully controlled by introduction of Oryctes rhinoceros nudivirus (OrNV) as a classical biological control agent. However, CRB-G is resistant to all currently available isolates of OrNV.

The selected applicant will:

- Participate in foreign exploration for isolates of OrNV which are pathogenic to CRB-G. Foreign travel will be required.
- Perform bioassays to evaluate OrNV isolates as biocontrol agents for CRB-G
- Propagate promising OrNV isolates for autodissemination
- Author and co-author reports and peer-reviewed publications documenting research results

CLEARANCES

- College transcripts should be submitted with application
- Upon selection, the applicant must submit PPD, police and court clearances to UOG HRO.