

Final Report

USDA APHIS PPQ Biological Control Project 13-8515-1555-CA

**Efficacy of Entomopathogenic Fungus for
Biological Control of Coconut Rhinoceros
Beetle (CRB) on Guam and DNA
Profiling of Asia/Pacific CRB Populations
with Respect to Virus Susceptibility**

Prepared by

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December 2, 2015



In a Nut Shell

- Based on 21 day mortality data for 1,466 CRB collected from 75 sample sites during the Guam GMF survey, it is estimated that between 10% and 38% are being killed by GMF infection. We did not see a positive correlation between GMF mortality and rainfall (For more information, see Appendix 1).
- We are now referring to the Guam CRB genotype (CRB-G) as a new invasive biotype which has escaped from biological control by *Oryctes rhinoceros* nudivirus (OrNV). Sean Marshall made a presentation on this topic at the annual meeting of the Society for Invertebrate Pathology in August 2015 (see Appendix 3). CRB-G has been detected in Guam, Hawaii, Palau, and Port Moresby, Papua New Guinea, and Honiara, Solomon Islands (For more information, see Appendix 2). This survey will continue with the objectives of finding the geographical origin for CRB-G and OrNV isolates pathogenic for this biotype when and if additional financial support becomes available.
- In a last ditch effort to find OrNV pathogenic to CRB-G, we made a 'witches brew' by making a slurry of all CRB cadavers and virus samples left over from previous laboratory bioassays. CRB adults which swam in this slurry for 30 minutes had a significantly higher mortality than those which swam in water. Beetles which died in this were run through a second 'witch's brew' experiment. In both experiments 21-day mortality was significantly higher in beetles treated with the slurry compared to those treated with water. Gut samples were preserved from beetles which died in these experiments and these will be tested for virus when and if additional financial support becomes available (For more information, see Appendix 4).

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1 Background

The coconut rhinoceros beetle (CRB), *Oryctes rhinoceros*, was first detected on Guam on September 12, 2007. If left uncontrolled, it is expected that the infestation will kill 50% of Guam's coconut palms as this is what happened within a few years following arrival of CRB in the Palau Islands during the 1940s.

The Guam Coconut Rhinoceros Beetle Eradication Project is attempting to eradicate CRB from Guam. In the event that eradication is not achieved, it seems prudent to establish density-dependent biological control to prevent an incipient population explosion which will result in high numbers of adults flying around, greatly increasing risk of accidental export of this major pest to other Micronesian islands, Hawaii, and elsewhere.

Note that the background paragraph, above, was written at the inception of this biocontrol project and it is already out of date:

- The Guam eradication project failed. Current efforts by the Guam Coconut Rhinoceros Beetle project are aimed at developing CRB integrated pest management to minimize damage, implementation of effective biocontrol, and minimizing risk of accidental transport of CRB to other locations.
- What we are now calling the Guam CRB biotype was detected in new invasions on Oahu, Hawaii in December 2013 and in Honiara, Solomon Islands in January 2015. This biotype is genetically distinct and it is apparently resistant to oryctes nudivirus (OrNV), the preferred biocontrol agent for CRB.

1.1 Green Muscardine Fungus (GMF), as a Biocontrol Agent for Guam's CRB Population

Metarhizium majus, an entomopathogenic fungus commonly referred to as green muscardine fungus (GMF), was imported from the Philippine Coconut Authority as an alternative to OrNV. Following laboratory bioassays which indicated that GMF is highly pathogenic to Guam's CRB, field releases began in September, 2011 using direct application of spores to known CRB breeding sites and also by autodissemination. Adult males caught in pheromone traps are dusted with GMF spores and released. To date, 20 15 kg shipments of GMF spores have been imported from the Philippines to Guam and released at many natural and artificial breeding sites. GMF has also been autodisseminated by dusting trapped male beetles with spores and releasing them.

There were no observations of fungal pathogens killing CRB immatures or adults on Guam prior to our first releases in 2011. *M. majus* has apparently established on Guam as a self-sustaining biocontrol agent as evidenced by metarhized beetles in areas remote from spore release sites. One objective of this grant is to evaluate the efficacy of GMF as a biocontrol agent on Guam by measuring the proportion of beetles killed by the fungus in treated and untreated breeding sites.

1.2 *Oryctes* nudivirus (OrNV) as a Biocontrol Agent for Guam's CRB Population

Given that a species-specific entomopathogenic virus, the *Oryctes* nudivirus (OrNV), has proven effective elsewhere in the Pacific, the Eradication Project imported eight strains of this virus from AgResearch New Zealand from Dr. Trevor Jackson's lab where it is produced in insect tissue culture. The Project planned to autodisseminate this virus by infecting and releasing adult beetles. However, repeated laboratory bioassays indicated that none of the virus strains from NZ were pathogenic to Guam's beetle. An additional virus isolate, recovered from the guts of infected beetles in Fiji, also failed to affect Guam beetles. There are two possible reasons for this failure:

- Guam's beetles may be resistant to the virus
- The virus strains, produced in insect cell culture, may have lost potency

AgResearch New Zealand has been awarded a contract supported by USDA-APHIS funds (12-8515-1555-CA) to find out why OrNV has proven ineffective as a biocontrol agent for Guam's CRB and to search for virus strains which are highly pathogenic for Guam's CRB population.

Sean Marshall (AgResearch, New Zealand) has conducted preliminary CO1 DNA barcoding on CRB specimens from Guam. Sequences from this pilot investigation confirmed that CRB collected from Guam is the same species (i.e. *Oryctes rhinoceros*) as is present in Fiji, PNG, and Samoa. Further analysis of the CO1 sequences revealed that a specific mutation correlated with only the Guam insects (Table 1). We hypothesize that this difference might be linked to apparent virus resistance in the Guam population. If this proposal is funded, we will build a CO1 barcode library for CRB populations within the Asian-Pacific region. We plan to barcode CRB DNA samples from Palau, the Philippines, Fiji, Samoa, Papua New Guinea, Malaysia, Indonesia, and possibly elsewhere.

Relative virus susceptibility Guam versus other CRB populations is being assessed under a previous grant (12-8515-1555-CA). DNA barcodes may be useful in determining the source of the Guam CRB population and the source of other CRB incursions. Half of the funding from this grant (\$20k) is being used to support collaboration with Dr. Marshall at AgResearch New Zealand with respect to a search for an effective OrNV biocontrol agent and CRB genotyping.

2 Objectives

2.1 Estimate the Proportion of Guam's CRB Population Killed by the Biocontrol Agent, *Metarhizium majus*

Following the discovery that none of eight available strains of OrNV were pathogenic for Guam's CRB population, GMF was introduced from the Philippines for a classical biological control. GMF has been widely disseminated and is now well-established on Guam.

Table 1: *Oryctes rhinoceros* specimens with CO1 barcoding sequences positive for the Guam or the Fiji genotype.

Specimen Location	n =	Guam genotype	Fiji genotype
Guam	15	11	0
Fiji	6	0	6
Samoa	7	0	7
Papua New Guinea	5	0	5

Our objective is to measure the impact of GMF on the Guam CRB population in terms of the proportion of insects killed. We will also measure persistence of GMF infectivity after application. We need this information to determine if GMF application is a useful component of integrated pest management for CRB.

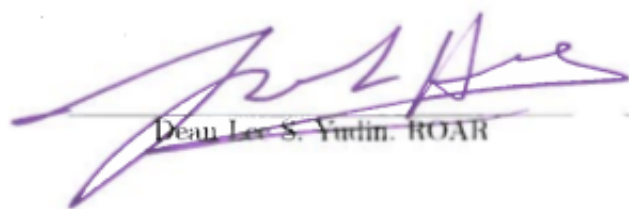

2.2 Develop DNA Profiles for CRB Populations in Asia and the Pacific with Respect to Virus Susceptibility

ONV has been effective in reducing CRB population levels and keeping them at low levels elsewhere in the Pacific. However, from bioassay results to date on the Guam CRB population has so far proven recalcitrant to currently available OrNV strains, which are able to cause disease in other populations. Our objective is to obtain samples of CRB from several geographic regions throughout the Asia-Pacific region to determine if the Guam CRB population is unique from those of other areas reporting OrNV susceptible CRB populations. Results from this analysis will determine if there is indeed a correlation between different CRB populations and OrNV susceptibility, and will also provide insight into the likely original source of the Guam CRB population. This information provides an important base for developing future biosecurity policies and CRB eradication/management efforts.

3 Collaboration

- Dr. Aubrey Moore, University of Guam (Principal Investigator)
- Dr. Sean Marshall, Dr. Trevor Jackson, AgResearch, New Zealand
- Mr. Roland Quitugua, University of Guam
- Dr. Russell Campbell, Guam Department of Agriculture
- Dr. Maclean Vaqalo, Secretariat of the Pacific Community, Land Resources Division, Fiji

4 Signatures

 _____ Dean Lee S. Yudin. ROAR	 _____ Date
 _____ Vernon Harrington. ADODR	 _____ Date

Appendix 1 - Guam Green Muscardine Fungus Survey

Guam GMF Survey

Aubrey Moore, Ian Iriarte and Roland Quitugua

December 2, 2015

This technical report documents a survey undertaken to investigate impacts of the biological control agent, green muscardine fungus (GMF), *Metarhizum majus*, on the Guam population of coconut rhinoceros beetle.

One thousand four hundred and sixty-six (1,466) beetles were sampled from 77 breeding sites between October 30, 2014 and May 26, 2015 and held individually in a soil/steer manure blend for 21 days. Thirty-eight percent of beetles died and 10% showed signs of fungal infection.

Methods

Mason jar Preparation

1. Wash each Mason jar and lid with soap and water. Set aside and let dry.
2. Prepare 80% alcohol solution in a medium to large container.
3. Immerse each jar and lid in the alcohol solution. Set aside and let it dry.
4. Prepare steer moist manure mixture. Note: If manure is too dry, water must be added so that it is damp. If already moist, no need to add water.
5. Once Mason jar is dry, fill each jar with moist steer manure. Fill jars about half full.
6. Cover jar with lid, puncture a hole, place a cloth or piece of napkin on top, and seal with metal ring (repeat for each jar).

Collection

1. Divide the desired surveying area into 2 parts and have one group per area to collect samples. Each group will get an equal number of jars, and each group will collect samples from areas that have been introduced to GMF and sites that have not.
2. Once samples are collected, all the jars are put into an incubator for 21 days.
3. After 21 days, each jar is inspected

4. All dead CRB are put into a small storage bag, labeled, and put into the freezer to be preserved.
5. All data are recorded and put into a spread sheet.

Rainfall Data

Rainfall data for the Guam International Airport (station ID 41406) were downloaded from the Applied Climate Information System using the ACISLoader python script.

Analysis

Data were stored in an Excel spreadsheet, 'GMF Survey data.xlsx'. Analysis was done using an IPython Notebook, 'Guam GMF Survey'.

Results and Discussion

Mortality from GMF Infection

- 21-day mortality for CRB sampled from 99 locations ranged from 0% to 100% with a mean of 38%.
- The proportion of CRB which produced fungal spores or hyphae within 21 days ranged from 0% to 55% with a mean of 10% (Fig. 1).
- Based on the above mortality data, we can estimate that between 10% and 38% of CRB are being killed by GMF infection.

Correlation with Rainfall

Correlation between mortality and cumulative rainfall during 90 days prior to sample date was insignificant (Figs. 2 and 3) (Pearson's correlation coefficient = -0.0673; $p = 0.6126$).

This result was unanticipated. We expected to see a positive correlation between mortality and rainfall under the hypothesis that GMF would be more prevalent during rainy periods.

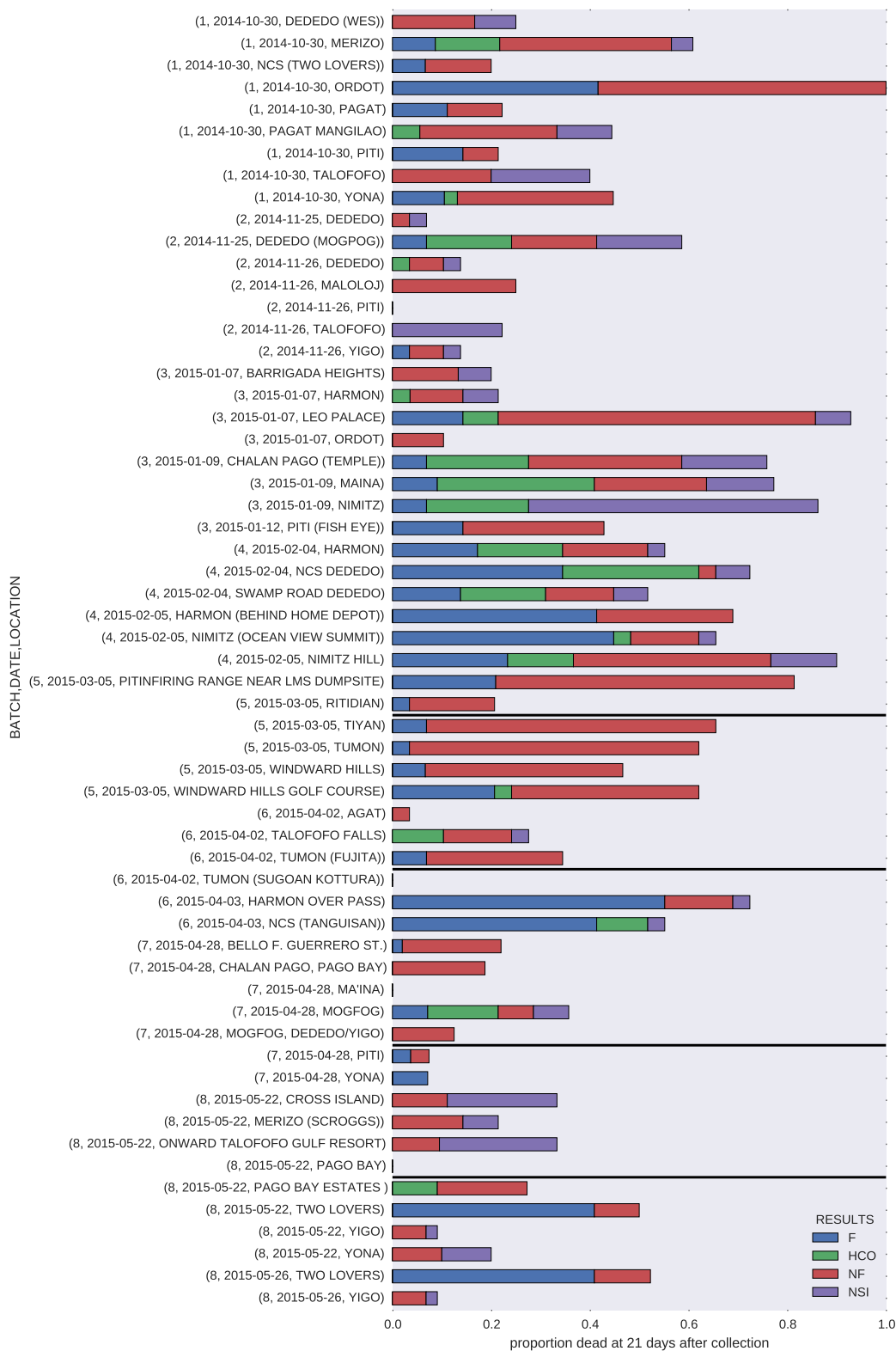


Figure 1: Proportion of insects dead at 21 days after collection. Legend: F - fungus evident; HCO - head capsule only was found; NF - no fungus evident; NSI - no sign of insect.

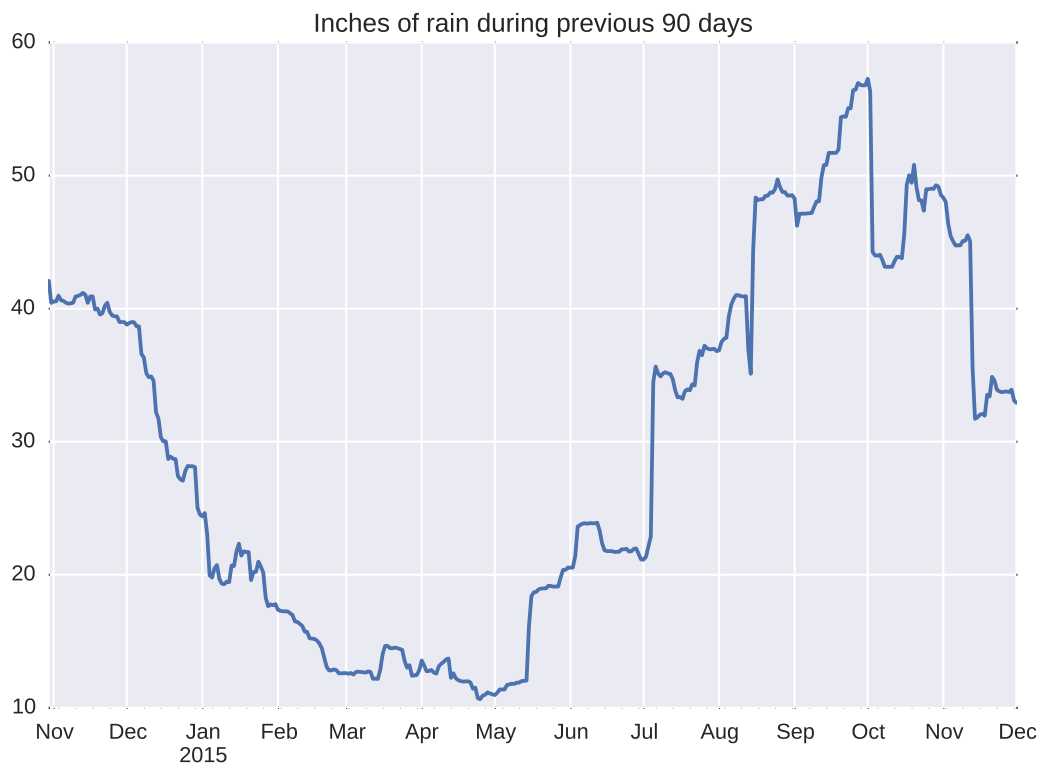


Figure 2: Accumulated rainfall in inches during the previous 90 days.

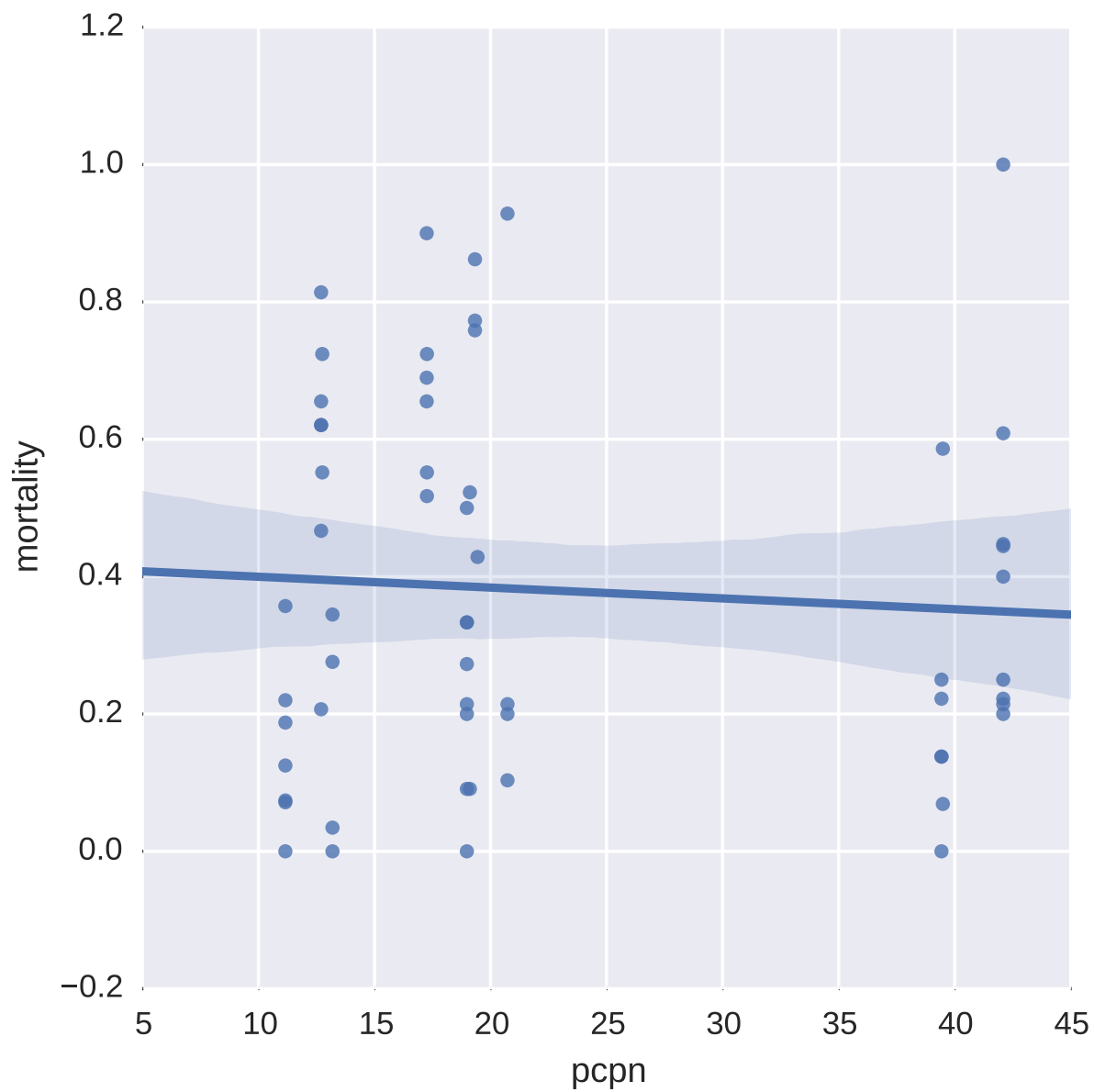


Figure 3: Correlation between mortality and accumulated rainfall in inches during the previous 90 days. (Pearson's correlation coefficient = -0.0673; $p = 0.6126$).

Appendix 2 - DNA Profiling of Asia/Pacific CRB Populations with Respect to Virus Susceptibility

DNA Profiling of Asia/Pacific CRB Populations with Respect to Virus Susceptibility

Sean D.G. Marshall

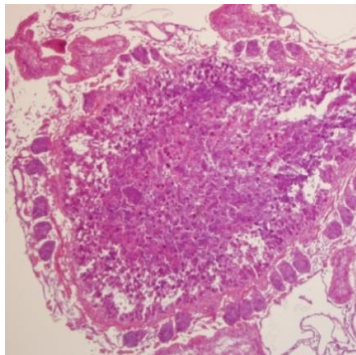
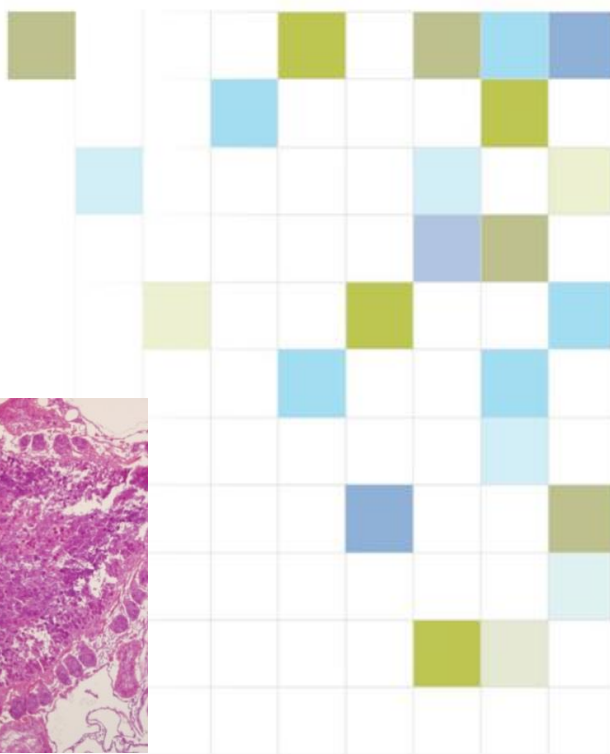
August 2015

Final Report for University of Guam

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1. EXECUTIVE SUMMARY

Project Summary

Oryctes nuditvius (OrNV) has been effective in reducing coconut rhinoceros beetle (CRB) population levels and keeping them at low levels elsewhere in the Pacific for over 40 years. However, to date the Guam CRB population has so far proven recalcitrant to currently available OrNV strains, which are able to cause disease in other populations. The objective of this project was to obtain CRB samples from several geographic regions throughout the Asia-Pacific region to determine if the Guam CRB population is distinct from OrNV susceptible CRB populations.

A library of DNA profiles was built from DNA extracted from CRB tissue specimens collected from several geographic locations within the Asian-Pacific region (Papua New Guinea, Samoa, Fiji, Guam, Hawaii, Palau, Malaysia, India, Diego Garcia). This was facilitated by our current collaborators (Secretariat of the Pacific Community, Malaysian Palm oil Board, Papua New Guinea Oil Palm Research Association, Biosecurity Solomon Islands, Fiji Ministry of Agriculture and Primary industries, Samoa Ministry of Agriculture and Fisheries, Guam Department of Agriculture, Hawaii Department of Agriculture) and through our own extensive science networks. Standard DNA analysis techniques (PCR, sequencing, RFLP) were used to catalogue and compare CRB DNA collected from the various locations. This information was compared to our knowledge on virus susceptibility and used to identify likely sources for the resistant Guam CRB population (CRB-G haplotype) and movement of this population around the Asia-Pacific region.

Results from our analysis have demonstrated the Guam CRB population is indeed genetically distinct from other CRB populations generally found elsewhere in the Pacific region. Additionally, we have found that the CRB-G haplotype has also spread to four other regions in the the Pacific, and and this haplotype correlates with reported levels of severe palm damage and possible OrNV resistance. While our finding did not identify the endemic source of the CRB-G population, it did narrow the range of future areas to look for the original source of the Guam CRB population. This information will better inform the nature of the Guam population and will assist in directing efforts towards identifying locations to search for alternative OrNV isolates that are more effective against Guam-like CRB populations. This information also provides an important base for developing future biosecurity policies and CRB eradication/management efforts.

2. BACKGROUND

The coconut rhinoceros beetle (CRB), *Oryctes rhinoceros*, was first detected on Guam on September 12, 2007. If left uncontrolled, it is expected that the infestation will kill 50% of Guam's coconut palms based on the impact following arrival of CRB in the Palau Islands during the 1940s. The Guam Coconut Rhinoceros Beetle Eradication Project is attempting to eradicate CRB from Guam. In the event that eradication is not achieved, it seems prudent to establish density-dependent biological control to prevent a population explosion which will result in high numbers of flying adults, greatly increasing risk of accidental export of this major pest to other Micronesian islands, Hawaii, and elsewhere.

The objective of this project was to obtain CRB samples from several geographic regions throughout the Asia-Pacific region to determine if the Guam CRB population is unique from those of other areas reporting OrNV susceptible CRB populations.

3. METHODS

3.1 Diagnostic methods for identifying *Oryctes rhinoceros* haplotype

Collection of CRB tissue for DNA extraction

Information on CRB collection, dissection and tissue preservation for DNA analysis is found in Appendix 8.2 "Collection of *Oryctes rhinoceros* tissue for analysis". Briefly, tissue samples were obtained from live CRB specimens that were collected from several geographic locations around the world. Specimens were collected using standard traps (bucket, vane, or similar) fitted with the CRB attractant, Oryctalure (www.chemtica.com). Tissue samples from live CRB specimens were 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 or 2 - 4 legs was submerged in monopropylene glycol (PPG), and stored at -20°C until ready to ship (or extract DNA).

PCR-RFLP method for detecting the 'Guam' CRB haplotype

DNA was extracted from CRB tissue using a Genomic DNA Isolate kit (Bioline). 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, publication in progress). 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 (UVItch). 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 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 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.

DNA sequence analysis of the *COI* gene barcode region from CRB specimens

DNA was extracted from CRB tissue using a Genomic DNA Isolate kit (Bioline). The primers used to amplify a region of the *COI* genome were universal barcode: LCO1490 (5'-GGTCAACAAATCATAAAGATATTG -3') and HCO2198 (5'-TAACTTCAGGGTGACCAAAAAATCA -3') (Folmer *et al.*, Simon *et al.*, 2006). Each 50 µl PCR reaction constituted 0.25 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 LCO1490 (10 µM), 0.5 µl HCO2198 (10 µM), 2 µl diluted (1 in 100) 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), 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%, 0.5xTBE), stained with RedSafe (iNtRON Biotechnology) and fluorescence visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). Successful PCR products were sent to MacroGen (www.macrogen.com/eng/) for purification and DNA sequencing. DNA was sequenced in both directions using *COI* barcoding primers LCO1490 and HCO2198 (Folmer *et al.*, 1994; Simon *et al.*, 2006).

DNA sequences returned from MacroGen were imported into the Geneious R8.0 (www.geneious.com, Kearse *et al.*, 2012) software package for further sequence manipulation and analyses. Partial *COI* sequences from individual specimens were trimmed, edited and contig assembled. Afterwards, sequences were aligned using MUSCLE algorithm integrated into Geneious 8.0 with default parameters. After removal of redundant sequences from the alignment, 35 representative of sequences remained from the dataset. Further trimming of the alignment was done to minimize end gaps, which yielded a 676 base pair fragment block of the *COI* gene. Tree construction was inferred from Bayesian phylogenetic analysis carried out in Geneious R8.0 over 1.0×10^6 generations using a HKY85 model with a Gamma rate variation.

4. RESULTS AND DISCUSSION

4.1 DNA Profiling of Asia-Pacific CRB Populations

Differentiation of CRB populations collected from several distinct geographic locations was initially carried out using the PCR-RFLP assay. This assay was developed to differentiate the Guam *Oryctes rhinoceros* haplotype (CRB-G) from other CRB populations such as CRB-S, which originally invaded Samoa and is commonly found throughout much of the Pacific. Figure 1 provides a representative example of PCR-RFLP results observed. Results to date have been summarized in Tables 1 and 2. The emerging trend suggests areas displaying low incidence of detectable OrNV correlated with reports of intermediate to high palm damage, while areas with moderate to high levels of detectable OrNV correlated with low to moderate levels of damage. Additionally, regions with confirmed populations of CRB-G correlated with severe levels of palm damage (e.g. Guam, Hawaii, Solomon Islands, Port Morsbey, and Palau). See Appendix 8.1 for a current map of the known distribution for *O. rhinoceros* and *Oryctes* nudivirus in the Asia Pacific region.

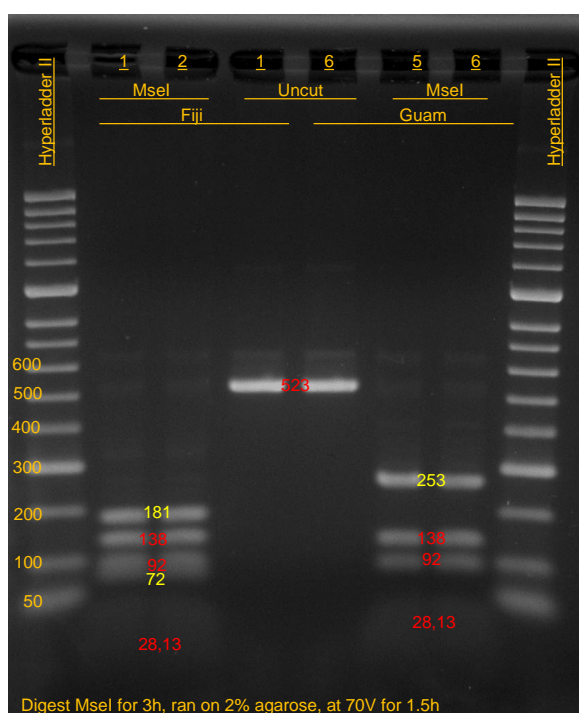


Figure 1: Representative results from a PCR-RFLP analysis demonstrating the difference between the Guam-like CRB genotype from the Fijian CRB genotype, which is the same as the commonly observed CRB genotype. Following PCR amplification of a 523 base pair (bp) fragment of the *COI* gene from *O. rhinoceros*, the *COI* amplicon was digested with the restriction enzyme *MseI* for 3 hours. The generated fragments were subsequently separated on a 2% agarose gel. Numbers on the figure next to the DNA bands indicate fragment size in base pairs. The DNA banding pattern is as follows: the two external lanes are a DNA size ladder, lanes 1, 2, 5, and 6 are *MseI*-digested DNA from PCR amplicons, and lanes 3 and 4 represent undigested DNA amplicons.

Table 1: Summary of *O. rhinoceros* 'biotype' and OrNV presence by country.

Country	Biotype	OrNV Present	% CRB-G	n=	% OrNV +	n=
PNG	CRB-S, CRB-G ¹	Yes	4.5	154	45.0	160
Fiji	CRB-S	Yes	0.0	38	48.7	39
Samoa	CRB-S	Yes	0.0	5	77.4	53
Malaysia	CRB-S	Yes	0.0	31	32.6	46
Diego Garcia	CRB-S	NA (legs only)	0.0	2	NA	0
India	CRB-S	Yes	0.0	4	50.0	4
Solomon Islands	CRB-G	No	100.0	10	0.0	10
Guam	CRB-G	No	100.0	17	0.0	17
Hawaii	CRB-G	No	100.0	14	0.0	14
Palau	CRB-S, CRB-G ²	Yes (both CRB-S, G)	72.7	11	72.7	11

¹, *O. rhinoceros* invaded the outer islands of PNG from 1942-1960. It was first detected in New Guinea at Port Moresby ca 2010, and has both CRB-S, and CRB-G present.

², *O. rhinoceros* first entered Palau in 1942, with CRB-G likely to be a second invasion (ca 2000's) due to the recent reports of increased levels of severe damage.

Table 2: Summary of *O. rhinoceros* 'biotype' and OrNV presence by region.

Country	Biotype	OrNV Present	% CRB-G	n=	% OrNV +	n=
PNG	CRB-S (except PoM)	Yes	4.5	154	45.0	160
New Ireland	CRB-S	Yes	0.0	105	48.7	113
West New Britain	CRB-S	Yes	0.0	26	28.6	28
East New Britain	CRB-S	Yes	0.0	13	53.8	13
New Guinea	CRB-S, CRB-G	Yes	70.0	10	33.3	6
Fiji	CRB-S	Yes	0.0	38	48.7	39
Viti Levu	CRB-S	Yes	0.0	25	65.4	26
Vanua Levu	CRB-S	Yes	0.0	10	10.0	10
Yasawa	CRB-S	Yes	0.0	3	33.3	3
Samoa	CRB-S	Yes	0.0	5	77.4	53
Upolo	CRB-S	Yes	0.0	5	77.4	53
Malaysia	CRB-S	Yes	0.0	31	32.6	46
Type A OrNV*	CRB-S	Yes	0.0	24	30.8	39
Type B OrNV*	CRB-S	Yes	0.0	7	42.9	7
Diego Garcia	CRB-S	NA (legs only)	0.0	2	NA	0
India	CRB-S	Yes	0.0	4	50.0	4
Kerala	CRB-S	Yes	0.0	4	50.0	4
Solomon Islands	CRB-G	No	100.0	10	0.0	10
Honiara	CRB-G	No	100.0	10	0.0	10
Guam	CRB-G	No	100.0	17	0.0	17
Hawaii	CRB-G	No	100.0	14	0.0	14
O'ahu	CRB-G	No	100.0	14	0.0	14
Palau	CRB-S, CRB-G	Yes (both CRB-S, G)	72.7	11	72.7	11
Aimeliik	CRB-S, CRB-G	Yes (both CRB-S, G)	62.5	8	75.0	8
Ngarraard	CRB-G	Yes	100.0	1	0.0	1
Airai	CRB-G	Yes	100.0	2	100.0	2

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

To further characterize the CRB populations, DNA sequencing was carried out on the *COI* gene barcode region, which encompasses the small region used in the PCR-RFLP haplotype assay employed above. Results from the multiple sequence alignment and phylogenetic analysis (see Figures 2 and 3) demonstrate there are informative differences among the CRB populations. In particular, the A→G transition centred on a *MseI* restriction site, which represents the diagnostic feature of the CRB-G haplotype. From the tree reconstruction, it appears that the CRB-G populations identified from Guam, Hawaii, Port Morseby, and Solomon Islands are from a single original source population. The CRB-G population detected in Palau has likely either hybridized with the original invasive CRB-S population or has arisen from a different source. Additionally, it is of interest to note that the non-CRB-G population commonly found in PNG is distinct from Fiji and Samoan populations, which fits with historical records from the pattern of CRB invasion within the Pacific. Together this data provides a useful basis to begin tracking the origin of the highly damaging CRB-G haplotype and also begins to lay foundations for more making informed choices in using particular biocontrol agent isolates for the future, including use of virus isolates effective against CRB-G.

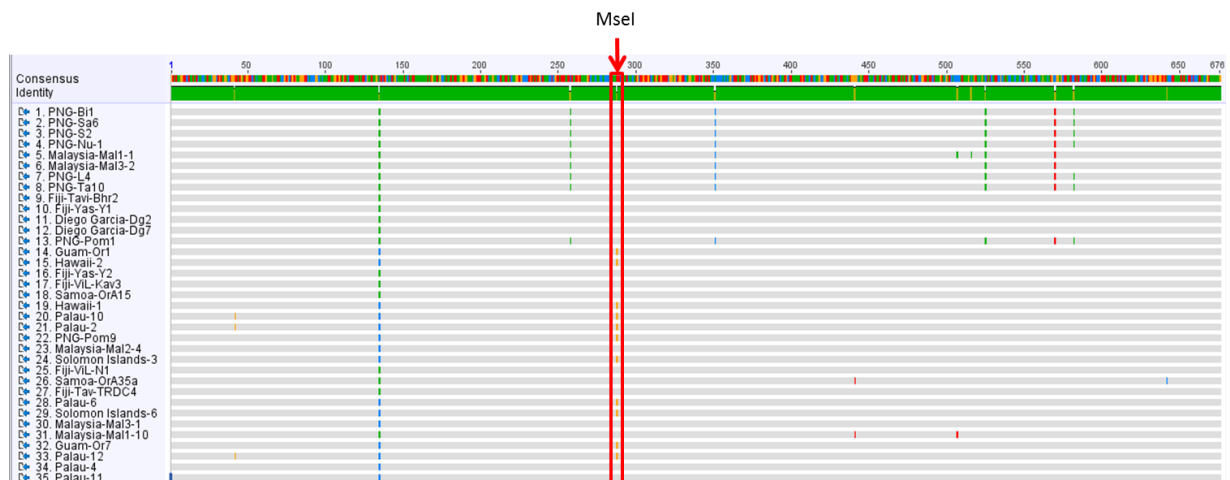


Figure 2: Multiple nucleotide sequence alignment of *O. rhinoceros* COI barcode region (676 bp fragment within the LCO1490-HCO2198 amplicon). Consensus sequence is displayed above alignment of 35 representative sequences selected based on geographic region and uniqueness. Red box highlights the position of the MseI restriction site for the PCR-RFLP assay. Coloured spots on individual grey bars indicate position of nucleotide differences.

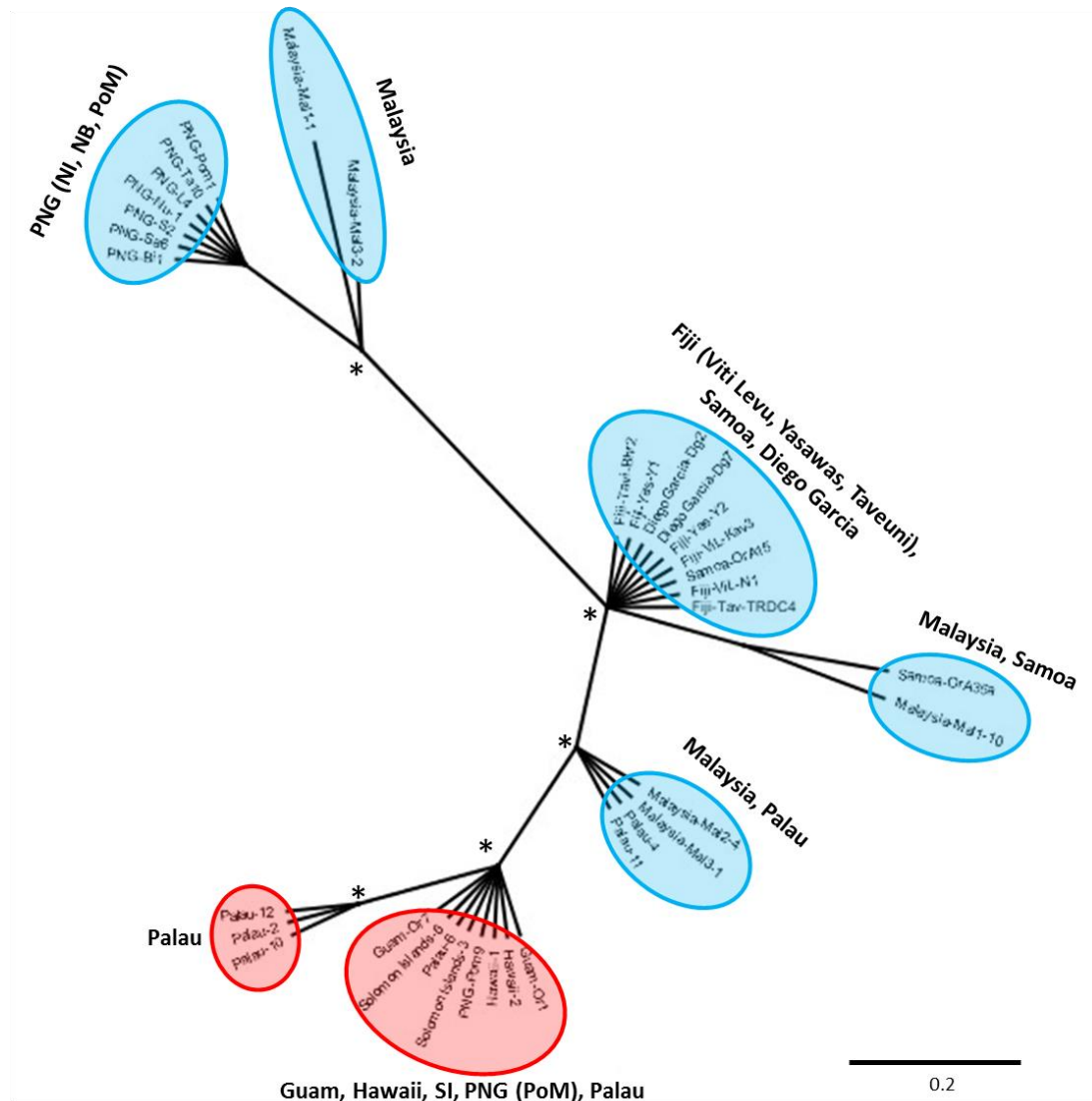


Figure 3: Phylogeny of *O. rhinoceros* partial COI region. The unrooted majority rule consensus tree includes 35 representative sequences from the dataset obtained from various geographic regions. The constructed tree was inferred from Bayesian phylogenetic analysis as implemented in Genious R8.0. Posterior probabilities (from 1.0×10^6 generations) are shown at branch nodes, where posterior probability scores of ≥ 0.95 are represented by *. Taxa highlighted in red correspond to the CRB-G haplotype based on the MseI PCR-RFLP assay, while the blue highlighted taxa do not.

Although further CRB specimens from native regions are required, at this point the evidence suggests that Malaysia is unlikely to be the origin for the CRB-G haplotype. However, the diversity observed within the Malaysia specimens demonstrates the potential diversity likely present within other native CRB ranges. This highlights the opportunity for future investigation into population-level variation found within the native CRB range, to identify both likely sources of new CRB invasions and areas likely to harbour effective isolates of biocontrol agents that are more effective against new invasive CRB populations.

5. RECOMMENDATIONS

Continue to collect CRB specimens to determine likely regions of origin for the CRB-G haplotype. This will assist with characterizing the haplotype in all known CRB populations, which will inform and coordinate pest management and biosecurity strategies within Asia and the Pacific regions. In particular, further collection of CRB from endemic regions (e.g. Asia and SE Asia) will be crucial as these populations will provide the greatest genetic diversity of both the host and pathogen species. This will allow isolation and selection of *Oryctes rhinoceros* virus isolates (and potentially other pathogens) that are effective against the CRB-G population.

6. ACKNOWLEDGEMENTS

We thank the following people for assistance in arranging collection and provision of some of the CRB tissue specimens used in this study: Dr Ramle Moslim (Malaysian Palm Oil Board); Aradhana Deesh and Apenisa Sailo (Fiji Ministry of Agriculture and Primary Industries); Dr Bob MacFarlane (Biosecurity Solomon Islands); Dr Aubrey Moore (University of Guam); Dr Robert Curtiss and Dr Darcy Oishi (Hawaii Department of Agriculture); Kuateman Tuapola (Samoa Ministry of Agriculture and Fisheries), Dr Maclean Vaqalo (Secretariat of the Pacific Community); Solomon Sar (Papua New Guinea Oil Palm Research Association), Dr Charlotte Pushparajan.

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8. APPENDICES

8.1 Supplementary information

Mapping of regional *Oryctes rhinoceros* information to improve management efforts

A regional database of distribution for CRB populations is being developed in collaboration with the Secretariat of the Pacific Communities (SPC) (funded via NZ MFAT SSDPF project CON/517/4/SSDPF “Biological Control Activity in Selected Pacific Island Countries”). The aim is to incorporate current information on damage severity, genotype, and control efforts to allow identification of regional trends which will guide prioritization for a regional strategy for deployment of pest control resources.

A distribution map for CRB location and OrNV release has been compiled (see Figure 4), which is useful for raising awareness of where potential new CRB invasions could arise, and what nations are involved in CRB management activities. Current CRB project information for: CRB biotype location (CRB population genotyping); virus status (OrNV virus presence in CRB populations); and distribution of palm damage (not yet integrated into the map) is ongoing. This has already provided SPC with valuable information in terms of identifying future sites for data collection within PNG, Samoa and Fiji (partner countries within the SSDPF project), and in highlighting regions where reintroduction of OrNV should be targeted. In future the map will also assist in providing guidance for prioritizing which CRB specimens should next be analysed by the CRB control team(s) as they continue to implement CRB control strategies and biosecurity programmes.

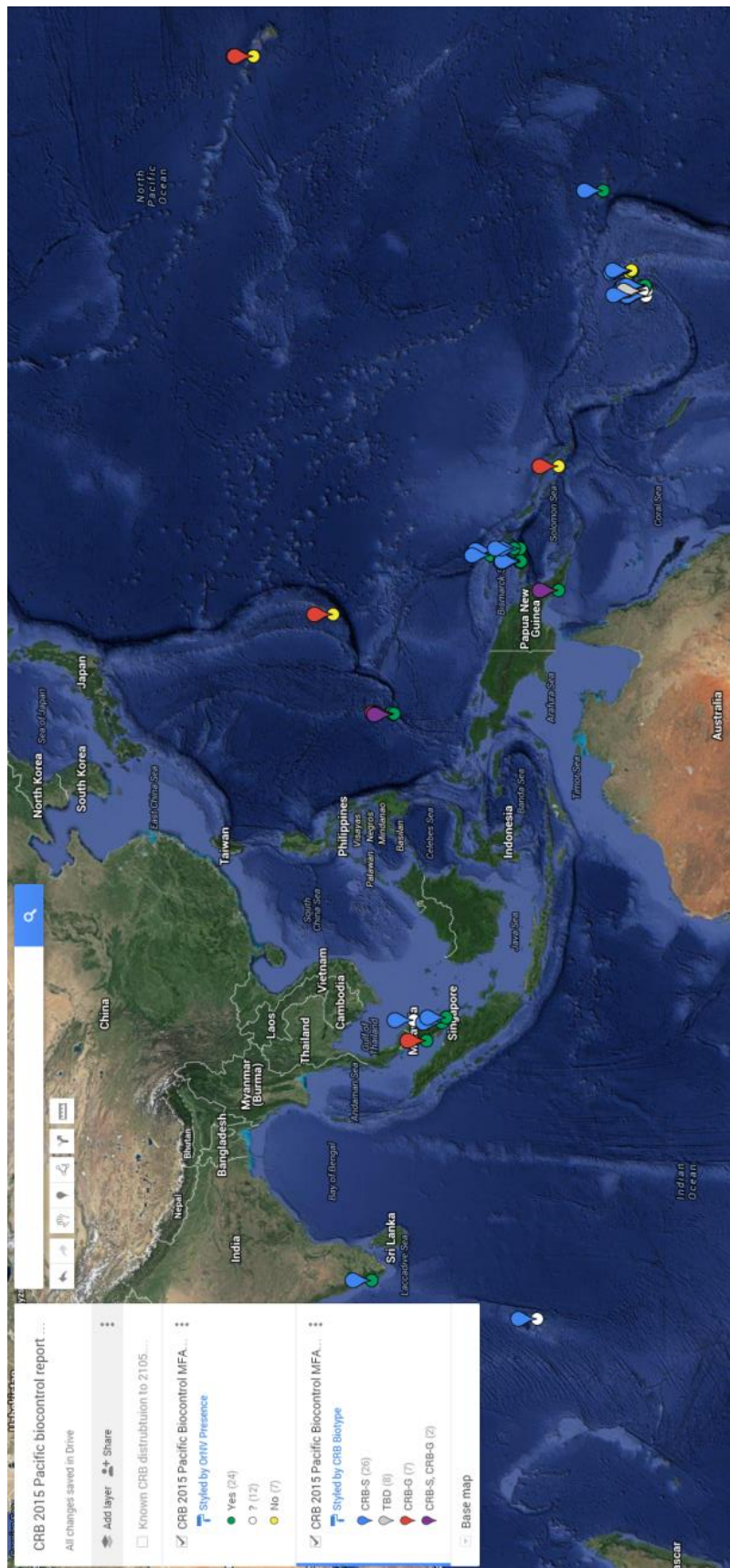


Figure 4: Distribution of *O. rhinoceros* and *Oryctes nudivirus* in the Asia Pacific region, June 2015. Balloons indicate *O. rhinoceros* biotype information (blue = CRB-S, red = CRB-G, purple = both CRB-S and CRB-G, grey = unknown/to be determined). Circles indicate status of *Oryctes nudivirus* (green = present, yellow = not present, grey = unknown/to be determine). This map was created using Google My Maps.

8.2 Protocol for collection of *Oryctes rhinoceros* tissue for diagnostic analysis

Version 22/04/2014

Prepared by: Sean Marshall (AgResearch, NZ)

This is an update to methods described in “Biological Control Of Rhinoceros Beetle In The Pacific Using *Oryctes* Virus Operational Protocols (Version B, April 2010)”, which will be revised accordingly.

Collection of *Oryctes rhinoceros* tissue samples for diagnostic analyses

- 1) Following collection of live beetles from pheromone traps or breeding sites, they should be placed into individual containers that are marked with the collection number. Storing insects individually reduces the risk of virus cross-contamination. The containers should be transported in a cool bin (so that insects will not overheat) to the laboratory where they must be stored in cool conditions until needed (e.g. for gut extractions). Beetles can be stored at 15-20°C for 2-3 days. If only DNA extraction is required, specimens can be stored frozen (keep at -20°C for long term storage), though take care to minimize thawing cycles prior to dissection as this will promote DNA degradation.
 - a. Beetles collected from pheromone traps – transfer of virus between beetles readily occurs within the confines of a pheromone trap, particularly if insects are left longer than overnight. If more than a 1 beetle has been caught in a trap, please record the trap number the beetles were from and the timing between trap inspections.
- 2) Record the location that individual *Oryctes rhinoceros* were collected from and include as much relevant detail as possible. For example:
 - a. A unique insect identification number, the location and trap number (e.g. GPS coordinates, nearest distinguishing landmark), the date of collection, gender of the beetle, dissection date, and the gut characteristics should be recorded.
 - b. If possible take photographs of the area for documentation of damage within the area
 - i. If time permits conduct a damage assessment for the area.

Below is an example datasheet for recording information

Insect ID	Site	Collection date	gender	Dissection date	Insect and gut condition	PCR result	Histology

- 3) Label each individual specimen tube using short codes that correspond with each individual beetle specimen. Use a permanent (non-smudge) marker pen for labelling.
- 4) Dissect out the gut as described in Chapter 3 of the document “Biological Control Of Rhinoceros Beetle In The Pacific Using *Oryctes* Virus Operational Protocols (Version B, April 2010)” (see below for a copy of this chapter).
 - a. During dissection, photograph the gut to document visual appearance. The photo can be used to cross reference with the written insect gut condition.
 - b. **Note:** If larvae have been collected, simply dissect out a portion of the upper thoracic region ('soft' part just behind the head, though the head itself can potentially be used as well). Please try to avoid the hind gut region as this contains a lot of microbes.
 - c. **Note:** (only relevant if genotyping insect species or populations!) if gut dissection is not feasible then remove whole legs from the body (a minimum of 2 legs, with leg muscle intact) as this will suffice for genotype analysis of the insect (however it will not be possible to reliably test for presence of *Oryctes* nudivirus infection)

Instructions for Gut Dissection

From: Chapter 3 of "*Biological Control Of Rhinoceros Beetle In The Pacific Using Oryctes Virus Operational Protocols (Version B, April 2010)*"

Dissection (Trevor Jackson)

Adult beetles must be dissected before disease diagnosis can be carried out. The method of storage of the dissected sample will depend on the diagnostic test being carried out.

Equipment for extraction of beetle gut

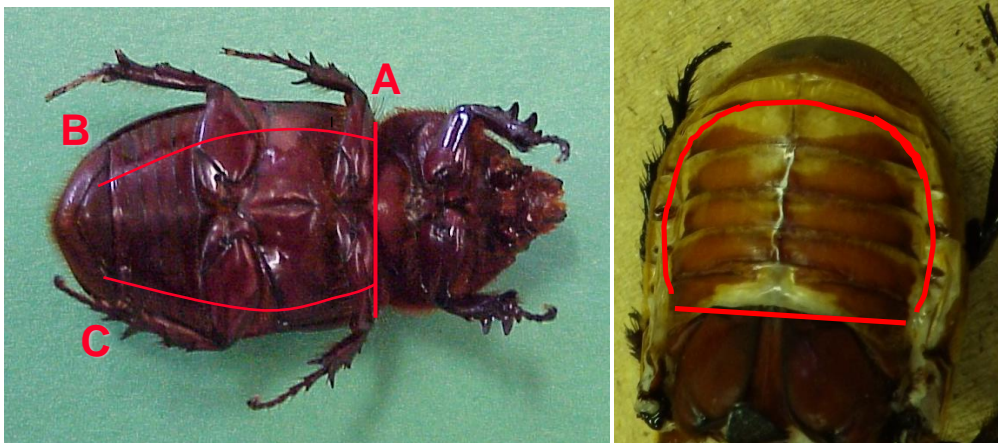
- Heavy and fine dissecting scissors
- Fine forceps
- Paper tissues (i.e. 'Kleenex', paper towels, or similar)
- Gloves (lab examination style)
- 0.1 M HCl* (wash bottle or spray bottle)
- 70-95% ethanol
- 2-ml tubes with O-ring
- Rubbish bag or bin for used tissues etc.

*inactivates DNA (e.g. on your equipment, bench surface), ethanol doesn't inactivate DNA.

Extraction of mid gut

Before starting, the bench surface and equipment should be wiped with 0.1 M HCl to inactivate any DNA and with 70% ethanol to remove any residues. Alternatively, if HCl and/or ethanol are not available, use a disinfectant wipe (e.g. bathroom cleaning wipe), but be sure to use a clean portion of the cloth with each wipe to minimize spread of contaminants.

To open the body cavity and expose the gut, the head and pronotum are removed by cutting across Line A, slightly to the posterior of the junction between the pronotum and the second thoracic segment with heavy scissors. The posterior section is then opened by cutting with fine scissors along lines B and C separating the dorsal and ventral cuticle.



Left is ventral view; Right is dorsal view (with head, elytra, and wings removed).

Heavy dissecting scissors are used to cut off the head and pronotum along line A.



To open up the abdomen it will be necessary to carefully insert the fine dissecting scissors along the line of the alimentary tract in order to cut through the wing muscles. The abdomen can then be opened along lines B and C. As an alternative to cutting along the ventral portion of the abdomen, one can carefully cut along the dorsal portion of the abdomen (as per the B and C lines). To avoid piercing the gut, angle the scissors up and away from the body.



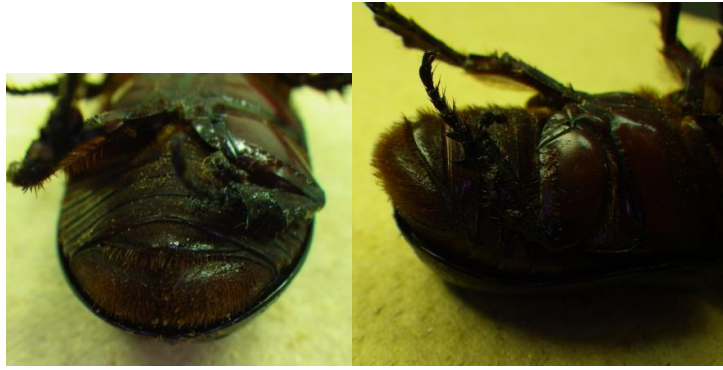
Fingers are used to pull apart the dorsal and ventral cuticle to expose the gut. Fine forceps can then be used to lift out the midgut. To avoid tearing the gut, carefully tease the gut from the surrounding tissue by gently pulling on the gut, releasing the pressure (before it breaks), grabbing another section with the forceps, and gently pulling on this new portion and again release the pressure; repeat these steps as required until the gut is free of the body. It may be necessary to cut the posterior attachment point where the gut meets the rectal opening.

Place gut sample into a 1.5-2mL tube containing propylene glycol (for DNA preservation), or FAA (for histology preservation)

Clean instruments and hands between each sample. Do not reuse paper tissues. If using disinfectant wipes, use a 'clean' area for cleaning action (e.g. wipe first scissor blade with one corner, wipe second blade with another corner). Also remember to wipe off gloves as these will be contaminated with beetle tissue from the dissection.

Gender determination

Female - Possess hair at the posterior end of the body. Horn on the head tends to be smaller than males, though this is not a reliable character. Release of eggs may be seen upon squeezing of abdomen, or presence of eggs may be observed within body cavity upon dissection.



Male - Posterior end of body is smooth (and may be shiny). Upon dissection, the male sex organs will be visible (distinctive hook shaped chitinous appendage)



Visual identification of infected midgut for isolation of OrNV from beetles

Dissection Equipment for extraction of beetle gut:

- Heavy and fine dissecting scissors
- Fine forceps
- Tissues (i.e. 'Kleenex', paper towels, etc)
- Gloves
 - M HCl* (wash bottle or spray bottle)
- 70-95% ethanol
- 1.7-2-ml tubes with O-ring
- Rubbish bag or bin for used tissues etc.

*inactivates DNA (e.g. on your equipment, bench surface), ethanol doesn't inactivate DNA

Extraction of mid gut

- Before you start wipe the bench surface and your equipment with 0.1M HCl to inactivate any DNA and with 70 % ethanol to remove any residues. Alternatively, if HCl and/or ethanol are not available, use a disinfectant wipe (e.g. bathroom cleaning wipe), but be sure to use a clean portion of the cloth with each wipe to minimize spread of contaminants.
- To open the abdomen and expose the gut, the head and pronotum are removed by cutting across Line A, slightly to the posterior of the junction between the pronotum and the second thoracic segment with heavy scissors. The posterior section is then opened by cutting with fine scissors along Lines B and C separating the dorsal (soft) and ventral (hard) cuticle.
 - To open up the abdomen it may be necessary to carefully insert the fine scissors along the line of the alimentary tract in order to cut through the wing muscles.
- Use fingers to pull apart the dorsal and ventral cuticle. The gut is now exposed. Use fine forceps pull out the midgut.
- Place gut sample in the tube containing propylene glycol (for DNA preservation)
- Clean instruments between each sample. Do not reuse tissues. However, if using disinfectant wipes, use a 'clean' area for cleaning action (e.g. wipe first scissor blade with one corner, wipe second blade with another corner). Also remember to wipe off gloves as these will be contaminated with beetle tissue from the dissection.
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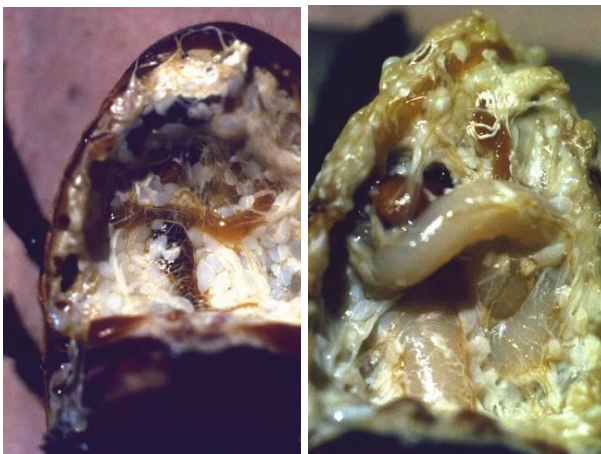


Figure 3: Dorsal view of *Oryctes rhinoceros* with elytra and wings removed and lines showing points to cut to expose gut material (left panel). Partially dissected abdomen of *O. rhinoceros*, displaying commonly observed healthy versus disease phenotypes (middle and right panels, respectively). The key aspects are: dark vs milky, thin vs swollen, empty vs full of organic matter.

Storage of gut tissue for diagnostics

The method of storage of the gut sample will depend on the diagnostic method being used (i.e. DNA vs histology). The midgut can be divided into two pieces (or more if required) to provide samples for both types of analysis.

- 1) For **DNA/PCR** analysis, the tissue sample should be immediately stored in 0.5-1ml of propylene glycol (enough to full immerse the tissue), gently mix to ensure tissue is fully submerged (if required; gentle mixing helps remove to remove air bubbles). Samples for DNA analysis are best stored at -20°C (preserved for several months to years). However, if a freezer is not available, storage at 4°C for 4 weeks or room temperature (up to 24°C) for 1 week is possible (note that some DNA degradation will occur). The propylene glycol solution has been pre-aliquoted into tubes within the collection kit
 - a. The advantage of using propylene glycol as a DNA preservative is that samples are considered to be preserved and are also non-toxic and so may be transported differently than for ethanol (see Transport of Samples, below).
 - i. If propylene glycol is not available, ethanol can be used to preserve tissue for DNA analysis. However, 95-100% ethanol should be used (70% is fine for a few days...not weeks), and airlines generally have strict policies on carrying ethanol (i.e. limited quantities and requires special packaging)
- 2) For **histology**, also see *Preservation of Tissue for Histological analysis* under the Appendices Section. Briefly, the gut sample should be immediately stored in FAA (5% formaldehyde, 2.5% glacial acetic acid, 50% absolute (99-100%) ethanol, 42.5% water), gently mix to ensure tissue is fully submerged (if required; gentle mixing helps remove to remove air bubbles). If FAA is not available, 10% neutral buffered formalin (10% NBF = 4% formaldehyde solution) can be used. Leave samples in fixative for a minimum of 24 hours. Samples may be left in fixative for several days to weeks prior to downstream processing.
 - a. The fixation solution can be pipetted (e.g. 1 ml aliquots into 1.7 ml specimen tubes) before dissections begin if a large number of samples are to be processed.
 - b. Both FAA and 10% NBF contain hazardous chemicals (see below). Please use appropriate protection when handling.
 - c. The advantages of FAA fixative are:
 - i. Tissues are fixed with minimal cell disruption and samples can be further processed for light or electron microscopy if required.
 - ii. Tissues can be stored in the fixative at room temperature without the samples becoming brittle or the need to change fixative (as is required for 10% NBF).

Shipment of Tissue Samples for Analysis

Sending the packages containing **only** propylene glycol as a preservative (for DNA/PCR analysis):

- 1) Place tissue samples (immersed in propylene glycol in a 1.7-ml screw-top tube with an O-ring seal) into a sealable plastic container and line with absorbent material (e.g. cellulose packing, thick paper towel or cotton wool) to soak up any spillage that may occur during transportation;
 - a. If a sturdy plastic container is not available, a sealable sturdy plastic bag may be used.
- 2) Place the sturdy container inside a plastic bag and seal.
 - a. If sturdy plastic container is not available, use a second a sealable sturdy plastic bag may be used.
- 3) Place the double sealed contents into a cardboard box (or other sturdy/crush proof material).
 - a. If a box is not possible use a padded courier bag or envelope, or bubble wrap the samples.
- 4) A description of contents must be prominently attached to the outside of the final shipping packaging (see appendices for example template). For example, if the specimens are packed into a box that is then placed inside a courier bag, the description of contents must be prominently attached to the outside of the courier bag so that it can be easily seen by the biosecurity inspectors at the NZ border. Mark the outside of the package as:

For scientific research.

Dead, preserved tissue specimens from *Oryctes rhinoceros* (coconut rhinoceros beetle).

Preservative used is mono-propylene glycol (PPG; non-restricted chemical).

Package contains *[insert # of vials]* vials containing preserved/non-viable specimens, and therefore are therefore considered to be non-regulated under the provisions of the Dangerous Goods Regulations.

- 5) Send package to the following address (see appendices for example template):

Sean Marshall
AgResearch Limited (Lincoln Research Centre)
cnr Springs Road and Gerald Street, Lincoln
Private Bag 4749
Christchurch 8140
New Zealand
T +64 3 321 8800 T (DD) +64 3 325 9935

- 6) An 'export invoice' or 'pro forma' invoice is often required to be filled in for international courier packages (see appendices for example template). This generally involves a separate piece of paper attached to the outside of the package that contain the receivers address and contact details, a description of goods (as per step 4 above), cost of goods, and the senders contact information. For research specimens the cost of goods please fill out a value <NZ\$10. The specimens themselves have no commercial/market value, so the 'cost of goods' is just the packaging.
 - a. Please contact your courier service for advice on whether an export invoice is required.
 - b. PPG is **not** classified as a hazardous substance under the Material Safety Data Sheet, and preserved/non-viable biological substances are considered to be non-regulated under the provisions of the Dangerous Goods Regulations.
- 7) Please note that while an import permit is not required for shipping **preserved** specimens into NZ (*all other specimens require an import permit*), the description of contents must be visible on the outside of the final packaging and must match the actual contents of the package. If the inspector counts 30 vials and the description says there are 20 vials or 35 vials, the package may be destroyed and/or legal action taken.

NB all non-preserved specimens require an import permit. For NZ biosecurity staff, preserved means all organisms associated with the specimens being sent are non-viable (e.g. dissected gut tissue will be non-viable, however it will contain associated microbes, and these microbes must also be dead!).

- a. Further information can be found in the document "Import Health Standard for the Importation into New Zealand of Nonviable Animal Specimens from All Countries" (see <http://www.biosecurity.govt.nz/files/ihs/inespeic.all.pdf>).

Sending the packages containing ethanol, FAA, and/or 10%NBF as preservatives:

The International Air Transport Association (IATA) Dangerous Goods Regulations permits the transport of small volumes of flammable and corrosive liquids in approved containers (LabPak-1). See web site: www.iata.org. The LabPak-1 shipping containers protect against potential leakage of the preserving chemicals from the sample tubes. They can be obtained from Thermofisher Scientific NZ Ltd (<http://www.thermofisher.co.nz>).

- 1) Wrap the sample tubes containing the tissue samples in absorbent material (e.g. cellulose packing, thick paper towel or cotton wool) and place inside a sealable plastic bag before placing inside shipping container. Bubble wrap or absorbent material can be used to stop movement of sample package inside the container.

- 2) A warning label 'Dangerous Goods in Excepted Quantities' must be displayed on the outside of the package (example of label shown below).). For example, if the specimens are packed into a box that is then placed inside a courier bag, the description of contents must be prominently attached to the outside of the courier bag so that it can be easily seen by the biosecurity inspectors at the NZ border. Complete the label with appropriate details as follows and glue on one side of the container that shows "infectious substance" biohazard label. When sending:
- For ethanol samples you only need to tick class 3 and write UN1170 and the proper shipping name is ethanol.
 - For FAA samples you need to tick class 3 write UN1170 and UN1198 with the proper shipping names being ethanol and <10%formaldehyde solution (respectively).
 - For 10% NBF samples (i.e. if FAA is not used as the histology fixative) you need only tick class 3 and write UN1198 and the proper shipping name is <10%formaldehyde solution.
- 3) A description of contents must be attached to the outside of the shipment. For LabPak-1 boxes this can be glued on the "documentation" panel. However, if the labpak-1 box is then placed inside a courier bag, the description of contents (and the dangerous goods label) must be prominently attached to the outside of the courier bag so that it can be easily seen by the biosecurity inspectors at the NZ border. Mark the outside of the package as:

For scientific research.

Dead, preserved tissue specimens from *Oryctes rhinoceros* (coconut rhinoceros beetle).

Preservative used is *[insert all that apply: ethanol, 4% formaldehyde, or FAA (50% ethanol / 5% formaldehyde / 2.5% acetic acid)]* with not more than 1ml of solution per vial.

Package contains *[insert # of vials]* vials containing specimens.

This volume qualifies as very small amounts of dangerous goods qualify and are therefore considered to be non-regulated under the "de minimis" provisions of the Dangerous Goods Regulations.

- 4) Send package to the following address:

Sean Marshall
AgResearch Limited (Lincoln Research Centre)
cnr Springs Road and Gerald Street, Lincoln
Private Bag 4749
Christchurch 8140
New Zealand
T +64 3 321 8800 T (DD) +64 3 325 9935

- 5) An 'export invoice' or 'pro forma' invoice is often required to be included for international courier packages. This generally involves a separate piece of paper attached to the outside of the package that contain the receivers address and contact details, a description of goods (as per step 4 above), and

cost of goods. For research specimens the cost of goods please fill out a value <NZ\$10. The specimens themselves have not market value, so the 'cost of goods' is just the packaging.

- b. Please contact your courier service for advice on whether an export invoice is required.

- 6) Please note that while an import permit is not required for shipping **preserved** specimens into NZ (*all other specimens require an import permit*), the description of contents must be visible on the outside of the final packaging and must match the actual contents of the package. If the inspector counts 30 vials and the description says there are 20 vials or 35 vials, the package may be destroyed and/or legal action taken.

NB all non-preserved specimens require an import permit. For NZ biosecurity staff, preserved means all organisms associated with the specimens being sent are non-viable (e.g. dissected gut tissue will be non-viable, however it will contain associated microbes, and these microbes must also be dead!).

- a. Further information can be found in the document "Import Health Standard for the Importation into New Zealand of Nonviable Animal Specimens from All Countries" (see <http://www.biosecurity.govt.nz/files/ihs/inespeic.all.pdf>).
- 7) As long as each ethanol or FAA preserved specimen is packaged: 1) within individual vials, 2) with each vial contains not more than 1ml of solution per vial, and 3) the single shipped package contains no more than or 100 ml of fixative solution, then this volume qualifies under the very small amounts of dangerous goods quantity and is therefore considered to be non-regulated under the "de minimis" provisions of the Dangerous Goods Regulations.

Appendices:

Preservation of Tissue for Histological analysis

Safety Information for Fixation Chemicals

By nature FAA and 10% NBF solutions contain harmful chemicals and should be handled with special precautions.

Formaldehyde is a dangerous chemical that is found in both the FAA and 10% NBF fixatives. Formaldehyde can be purchased as a liquid or in solid form. More information can be found at:

<http://swehsc.pharmacy.arizona.edu/exppath/resources/formaldehyde.php>.

Liquid forms of formaldehyde should be stored tightly sealed; since exposure to air encourages the oxidation of formaldehyde to formic acid (37% formaldehyde is usually shipped with 10-15% methanol to inhibit this change). Formaldehyde should be stored at

room temperature (cold temperatures encourage the formation of trioxymethylene with a resulting white precipitate). The shelf life of an unopened 37% formaldehyde stock solution is ca 1 year if stored in the dark at 20-25°C. Once opened, the solution should be used within 3-4 months. Therefore if possible purchase in small volumes. Solutions containing ca 4% formaldehyde should be used within 1-2 months, if not sooner. Suggested sources of formaldehyde include Sigma-Aldrich: #533998-500ML Formaldehyde histological grade, 37 wt. % in H₂O or Fluka: #47629 Formaldehyde solution.

An alternative to formaldehyde solutions is crystalline paraformaldehyde, which can be used to make up the required solutions, but it is hazardous to workers if recommended safety procedures are not followed. If you are not familiar with making solutions from the crystalline form, it is recommended that a premade formaldehyde solution is purchased.

Acetic acid is a component of the FAA fixative. Stock solutions of acetic acid (e.g. glacial or solutions >10%) are strong acids and should be handled with care. It is often purchased as glacial (meaning concentrated; e.g. Scharlau: #AC0352). Diluted acetic acid solutions can also be used as long as the final concentration in FAA is 2.5%.

Preparation of FAA Fixative

Prepare FAA in small volumes as needed (see Table below). Store at room temperature (20-25°C) and use within ca 2 months. The formaldehyde should be gently shaken before use to avoid a concentration gradient in the bottle.

Table: Directions for making 200 ml of FAA fixative (scale volume as required).

Stock Solution	Volume	Final Concentration
37% formaldehyde solution	25 ml	5%
Glacial acetic acid	5 ml	2.5%
95% ethanol	100 ml	50%
distilled water	70 ml	42.535%

Preparation of 10% NBF Fixative

An alternative fixative to FAA is 10% neutral buffered formalin (10% NBF; 10% formalin is an old name for a 4% formaldehyde solution). It may be easier and safer to buy premade 10% neutral buffered formalin. Use within printed expiry date if purchased premade or 3 months if self-made. Prepare self-made fixative in small volumes as needed (see Table below). Store 10% NBF at room temperature (20-25°C).

Table: Directions for making 100 ml of 10% NBF (scale volume as required).

Solution	Volume/weight
37-40% formaldehyde	10 ml
Sodium phosphate (monobasic) NaH ₂ PO ₄ x 1H ₂ O	0.4 g
Sodium phosphate dibasic (anhydrous) Na ₂ HPO ₄	0.65 g
Distilled water up to 1000 ml	ca 90 ml

Example Address and Description of Content label templates
(NB Revise wording as necessary, print, cut out, and attach to package.)

Template for shipping address and for Description of Contents:

<p><u>Ship to:</u> Sean Marshall AgResearch Limited (Lincoln Research Centre) cnr Springs Road and Gerald Street, Lincoln Private Bag 4749 Christchurch 8140 New Zealand T +64 3 321 8800 T (DD) +64 3 325 9935</p> <p><u>Receive from:</u> Name: Address: Phone:</p>

<p><u>Description of Contents</u></p> <p>For scientific research.</p> <p>Dead, preserved tissue specimens from <i>Oryctes rhinoceros</i> (coconut rhinoceros beetle).</p> <p>Preservative used is mono-propylene glycol (PPG; non-restricted chemical).</p> <p>Package contains [insert # of vials] vials containing preserved/non-viable specimens, and therefore are therefore considered to be non-regulated under the provisions of the Dangerous Goods Regulations.</p>

<p><u>Description of Contents</u></p> <p>For scientific research.</p> <p>Dead, preserved tissue specimens from <i>Oryctes rhinoceros</i> (coconut rhinoceros beetle).</p> <p>Preservative used is [insert all that apply: ethanol, 4% formaldehyde, or FAA (50% ethanol / 5% formaldehyde / 2.5% acetic acid)] with not more than 1ml of solution per vial.</p> <p>Package contains [insert # of vials] vials containing specimens.</p> <p>This volume qualifies as very small amounts of dangerous goods qualify and are therefore considered to be non-regulated under the “de minimis” provisions of the Dangerous Goods Regulations.</p>

Example of an Export Invoice

DESCRIPTION OF CONTENTS

<p>Sender: <i>Name</i> <i>Company</i> <i>Address</i></p> <p><i>Phone:</i> <i>Fax:</i> <i>E-mail:</i></p> <p>Consignees: <i>Name:</i> <i>Company:</i> <i>Address:</i></p> <p><i>Phone:</i> <i>Fax:</i> <i>E-mail:</i></p>	<p>Date:</p> <p>Comments: Package contains:</p> <p><i>For example:</i> For scientific research. Products are intended for research purposes in Consignees' laboratory only.</p> <p>Dead, preserved/non-viable tissue specimens from <i>Oryctes rhinoceros</i> (coconut rhinoceros beetle). Preservative used is mono-propylene glycol (PPG; non-restricted chemical).</p>
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TYPE OF PACKAGING	DETAILED DESCRIPTION OF CONTENTS	QUANTITY	COMMERCIAL VALUE*
Cardboard outer and Styrofoam inner	<p><i>Example...</i></p> <p>Dead, preserved tissue specimens from <i>Oryctes rhinoceros</i> (coconut rhinoceros beetle)</p> <p>Preservative used is mono-propylene glycol (PPG; non-restricted chemical).</p> <p>Specimens contained in sealable vials labelled as Fiji#1- 1 to 20.</p> <p>Preserved specimens are non-viable biological substances and are therefore considered to be non-regulated under the provisions of the Dangerous Goods Regulations.</p>	30 vials containing specimens	NZ\$10

I hereby confirm that the information contained in this statement is true and that the contents of this shipment are as stated above.

Signature: _____

Title: _____ **Date:** _____

Appendix 3 - Coconut Rhinoceros Beetle Genotyping

Abstract submitted to the Society for Invertebrate Pathology Annual Meeting, August 2015, Vancouver, Canada

A new invasive biotype of the coconut rhinoceros beetle (*Oryctes rhinoceros*) has escaped from biological control by *Oryctes rhinoceros* nudivirus

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The coconut rhinoceros beetle (*Oryctes rhinoceros*; CRB) is a major pest of coconut and oil palm, but the discovery of *Oryctes rhinoceros* nudivirus (OrNV) in the 1960s enabled the successful management of populations in Pacific Island Countries (PICs). 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 first time in ~40 years, CRB invasion into completely new areas has been reported in the Pacific, being detected first in Tumon Bay in Guam 2007, followed by Port Moresby in Papua New Guinea 2010, Honolulu in Hawaii 2013, and Honiara in Solomon Islands 2015. 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 were unexpectedly unsuccessful, raising the possibility that the CRB-G population that invaded Guam could be tolerant or resistant to the commonly applied OrNV isolates. Analysis of several CRB populations has demonstrated that the CRB-G biotype is also found in Hawaii, Palau, and recently (February 2015) in Port Moresby (PNG), with Honiara (Solomon Islands) still to be confirmed. We will discuss current results in relation to what is known about these new invasions and potential implications for the future.

Appendix 4 - Oryctes Nudivirus Witch's Brew Experiment

OrNV Witch's Brew Experiment 1: A Last Ditch Attempt to Find Virus Pathogenetic for the Guam Coconut Rhinoceros Beetle Genotype

Aubrey Moore, Ian Iriarte and Roland Quitugua

December 2, 2015

Bioassays of several isolates of *Oryctes nudivirus* provided by AgriResearch New Zealand failed to result in significant pathogenicity for the Guam CRB genotype. In a 'last ditch' attempt we made a 'witches brew' slurry containing all frozen dead beetles from previous bioassays plus frozen virus samples in vials. Forty adult beetles were forced to swim in the slurry for 30 minutes on January 22, 2015. A control group of 41 beetles were forced to swim in water. Beetles were checked weekly.

By April 10, 2015, mortality of the virus treated beetles (78%) was significantly greater than that of the control group (54%). Treatment mortality corrected for experimental control mortality by Abbott's formula was 51%.

Methods

Frozen, dead beetles from 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.

Analysis

Data were analyzed using an IPython notebook (file name = 'OrNV.ipynb'). Significance of differences in mortality were determined using a Fisher's exact test, and final mortality was adjusted using Abbott's formula.

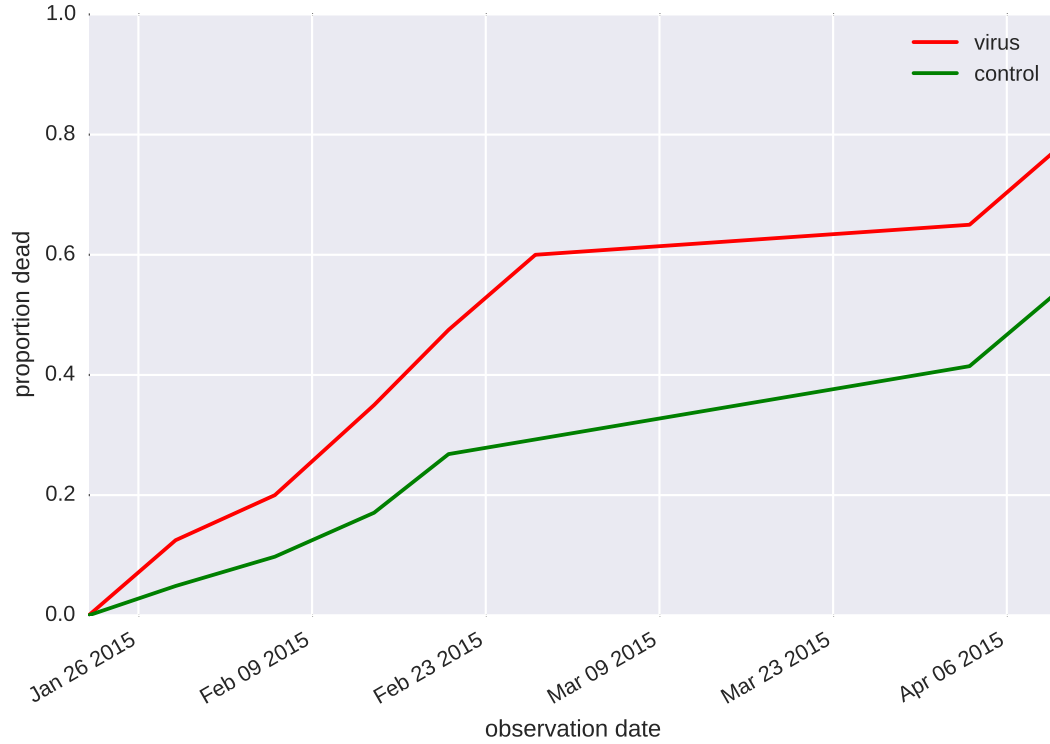


Figure 1: Cumulative mortality.

Results and Discussion

Cumulative mortality of virus-treated beetles (78%) on April 10 (Fig. 1) was significantly greater than that of control beetles (54%); ($p = 0.0005$; Fisher's exact test). Treatment mortality corrected for experimental control mortality by Abbott's formula was 51%.

OrNV Witch's Brew Experiment 2: A Last Ditch Attempt to Find Virus Pathogenetic for the Guam Coconut Rhinoceros Beetle Genotype

Aubrey Moore, Ian Iriarte and Roland Quitugua

December 2, 2015

In this experiment, we made a 'witch's brew' using frozen beetles which died in the 'Witch's Brew 1' bioassay. Sixty-one adult beetles were forced to swim in the slurry for 30 minutes on June 29, 2015. A control group of 59 beetles were forced to swim in water. Beetles were checked weekly.

By July 27, 2015, mortality of the virus treated beetles (75%) was significantly greater than that of the control group (48%). Treatment mortality corrected for experimental control mortality by Abbott's formula was 53%.

Beetles which died in this experiment were preserved and these will be tested for virus if and when additional funding becomes available.

Methods

Frozen, dead beetles from 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.

Analysis

Data were analyzed using an IPython notebook (file name = 'witch's brew 2.ipynb'). Significance of differences in mortality were determined using a Fisher's exact test, and final mortality was adjusted using Abbott's formula.

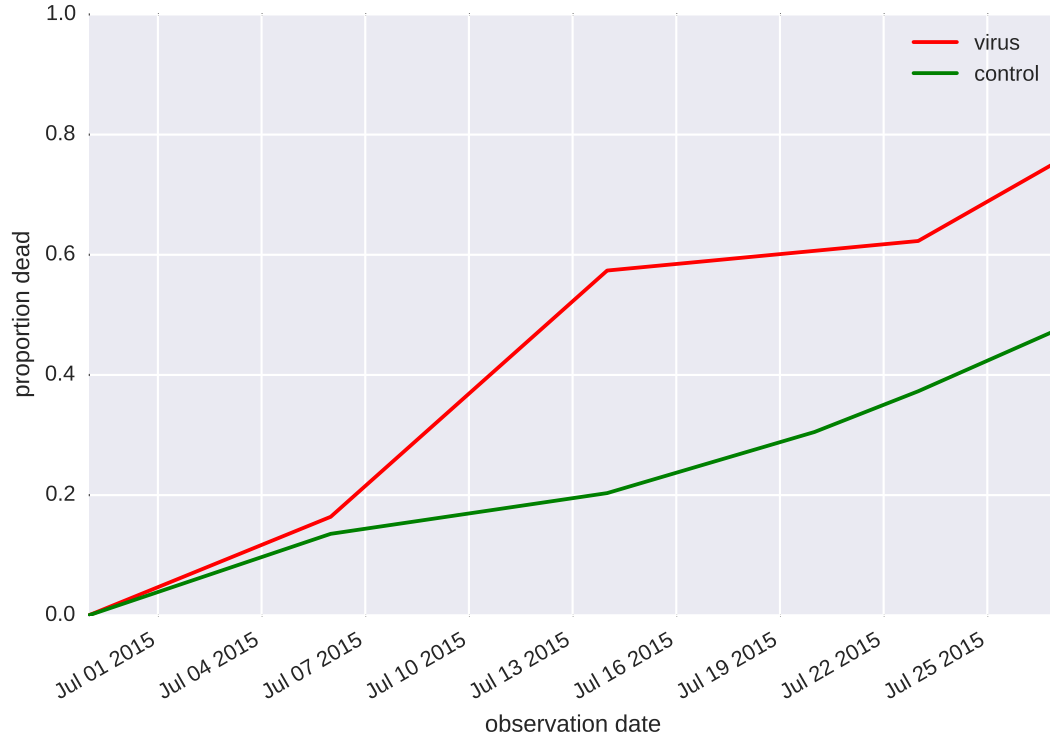


Figure 1: Cumulative mortality.

Results and Discussion

Cumulative mortality of virus-treated beetles (75%) on April 27 (Fig. 1) was significantly greater than that of control beetles (48%); ($p = 0.0014$; Fisher's exact test). Treatment mortality corrected for experimental control mortality by Abbott's formula was 53%.