Semiannual 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

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May 5, 2015

In a Nut Shell

- Based on 21 day mortality data of CRB collected from 42 sample sites during the ongoing Guam GMF survey, it is estimated that between 12% and 45% are being killed by GMF infection. This estimate will be refined when necropsies and microsopy are performed to look for distinctive *Metarhizium majus* spores. We did not see a positve 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 will make a presentation on this topic at the annual meeting of the Society for Invertebrate Pathology in August 2015. CRB-G has been detected in Guam, Hawaii, Palau, and Port Moresby, Papua New Guinea. Collection of CRB DNA samples from throughout Asia and the Pacific continue. (For more information, see Appendix 2).
- 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 experiment will be examined for signs of virus infection, and a second 'witches brew' experiment will be performed using a slurry made from these beetles. (For more information, see Appendix 3).

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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, Soloman Islands in January 2015.

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

Metarhizium majus, a species-specific 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 indicate that GMF is a 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. However, contribution of GMF to CRB population control is unknown. We will attempt 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. *Orcytes 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-Pacifc 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 AgReaserch 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 Approach

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

We will collect CRB larvae and adults from newly discovered breeding sites and from breeding sites which have been previously treated with GMF. Specimens will be individually reared in Mason jars containing commercial steer manure/soil mix for three weeks to see if they die or develop symptoms of infection by GMF or other pathogens. A total of 200 sites will be sampled.

Progress to Date: 80%

- Informal surveys show that GMF has spread naturally via autodissemination from treatment sites. It is very commonly found infesting all CRB life stages at breeding sites during periods of wet weather.
- Has been found infesting grubs feeding in detritus in the crowns of unmanaged coconuts.
- Formal surveys are almost complete.

3.2 Develop DNA Profiles for CRB Populations in Asia and the Pacific

We will obtain CRB DNA samples from several geographic locations within the Asian-Pacific region (currently targeting Palau, Philippines, Fiji, Samoa, Papua New Guinea, Malaysia, Indonesia, and others where we are able). This will be facilitated by our current collaborators (see below) and through our own extensive science networks. To ensure sufficient population diversity is captured, we aim to collect 20 specimens from two or more separate locations within a targeted nation

Standard DNA analysis techniques (PCR, sequencing, RFLP) will be used to catalogue and compare CRB DNA collected from the various locations. This information can be mapped back to our knowledge on virus susceptibility and to provide insight into the likely source for the Guam CRB population and movement of the insect around the Asia-Pacific region.

Initially we will target analysis to the CO1 gene, as we currently have validated protocols for this gene. However, to improve our ability to differentiate between CRB populations we will investigate the possibility of being able to incorporate other 'barcoding' genes into our analysis.

Progress to Date: 80%

- DNA samples from Diego Garcia and Hawaii were obtained and sequenced. The Hawaii DNA matches that from Guam genotype and the Diego Garcia DNA matches that from Fiji genotype. See tech report downloadable from http://guaminsects.net/anr/sites/default/files/CRB2014-02-12.pdf.
- DNA samples from Palau were obtained and sequenced. The Palau DNA is mixed: about half the beetle samples had the Fiji genotype and about half had the Guam phenotype.
- DNA samples have been submitted from Papua New Guinea and the Solomon Islands. Additional DNA samples have been submitted from Oahu, Hawaii.
- DNA samples from the Philippines will be collected by A. Moore during his vacation in May, 2015.

4 Timeline

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

- **April 2014 - July 2015:** Field survey and laboratory rearing of specimens
- **April 2014 - August 2015:** Data analysis, project review and final report preparation.

4.2 Develop DNA Profiles for CRB Populations in Asia and the Pacific

- **March 2013:** Send out requests for CRB collection assistance. Collaborators will be asked to send dry rhino beetle legs to Guam. Batches of DNA specimens will be labeled, catalogued, preserved to meet New Zealand biosecurity specifications and shipped to Sean Marshall for processing and sequncing.
- **April 2014 - December 2014:** Extract DNA from specimens and conduct CO1 barcoding analysis (on going, as specimens arrive; anticipate timeframe of 6 months for specimens to be collected and sent back for analysis)
- **January 2015:** Test and validate other candidate barcoding genes for inclusion in the analysis.
- **June 2015:** Analyze and interpret data
- **August 2015:** Project review and final report preparation.

5 Collaboration

- Dr. Aubrey Moore, University of Guam (Principal Investigator)
- Dr. Sean Marshall, Dr. Trevor Jackson, Dr. Alan Crawford, 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

6 Signatures

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

Appendix 1 - Guam Green Muscardine Fungus Survey

Guam Coconut Rhinoceros Project Technical Report
DRAFT (Experiment Still in Progress)

Guam GMF Survey

Aubrey Moore, Ian Iriarte and Roland Quitugua

May 1, 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.

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 to 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 sample 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

- To date, total 21-day mortality for each location has ranged from 0% to 100% with a mean of 45%.
- The incidence of fungal spores or hyphae observed for each location ranged from 0% to 55% with a mean of 12% (Fig. 1).
- Based on the above mortality data, we can estimate that between 12% and 45% are being killed by GMF infection. This estimate will be refined when necropsies and microsopy are performed to look for distinctive *Metarhizium majus* spores.

Correlation with Rainfall

There was a significant (but marginal) negative correlation between total mortality and cumulative rainfall during 90 days prior to sample date (Figs. 2) and 3) (Pearson's correlation coefficient = -0.3053; p = 0.0492).

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

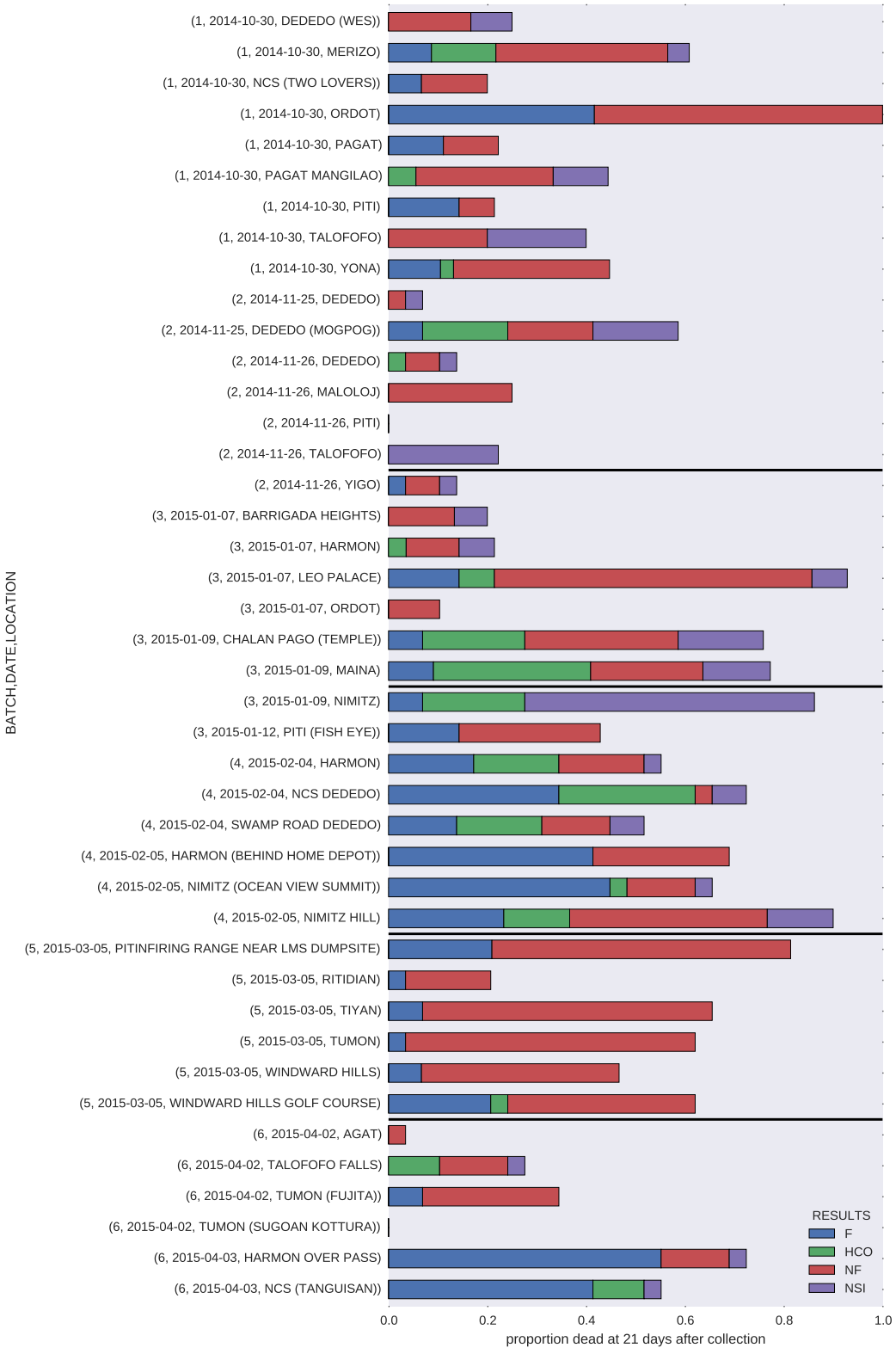

Figure 1: Proportion of insects dead at 21 days after collection. Legend: F - fungus evident; HCO - head capsule only was found; NF - no fungus evident; NSI - no sign of insect.

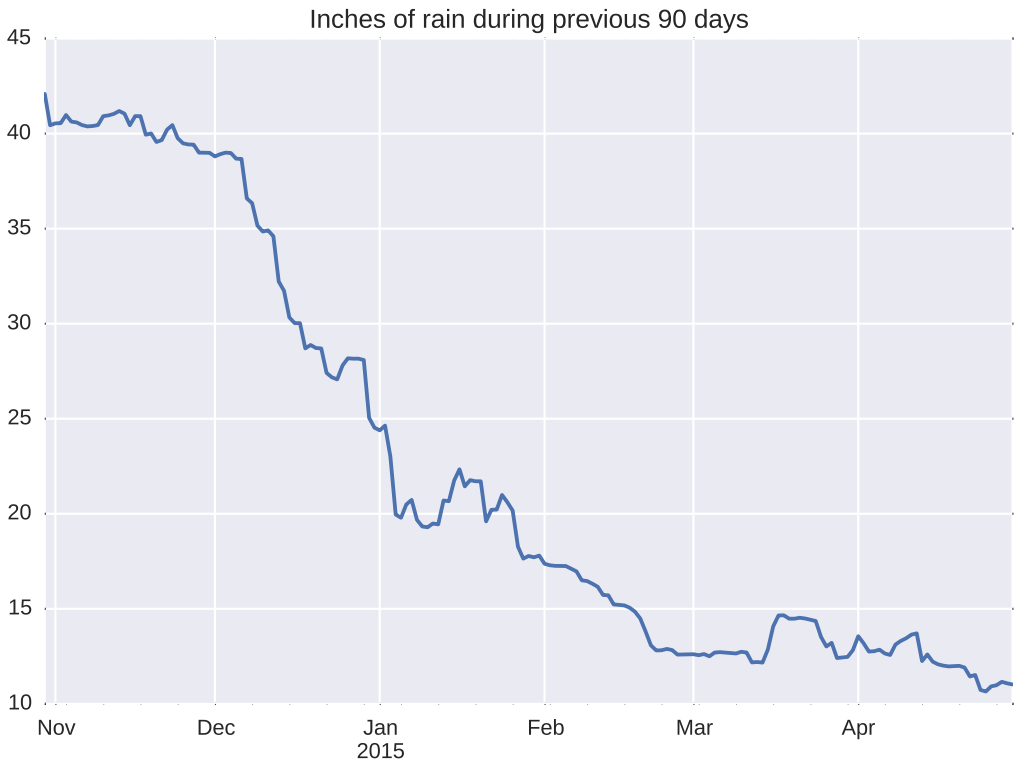

Figure 2: Accumulated rainfall in inches during the previous 90 days.

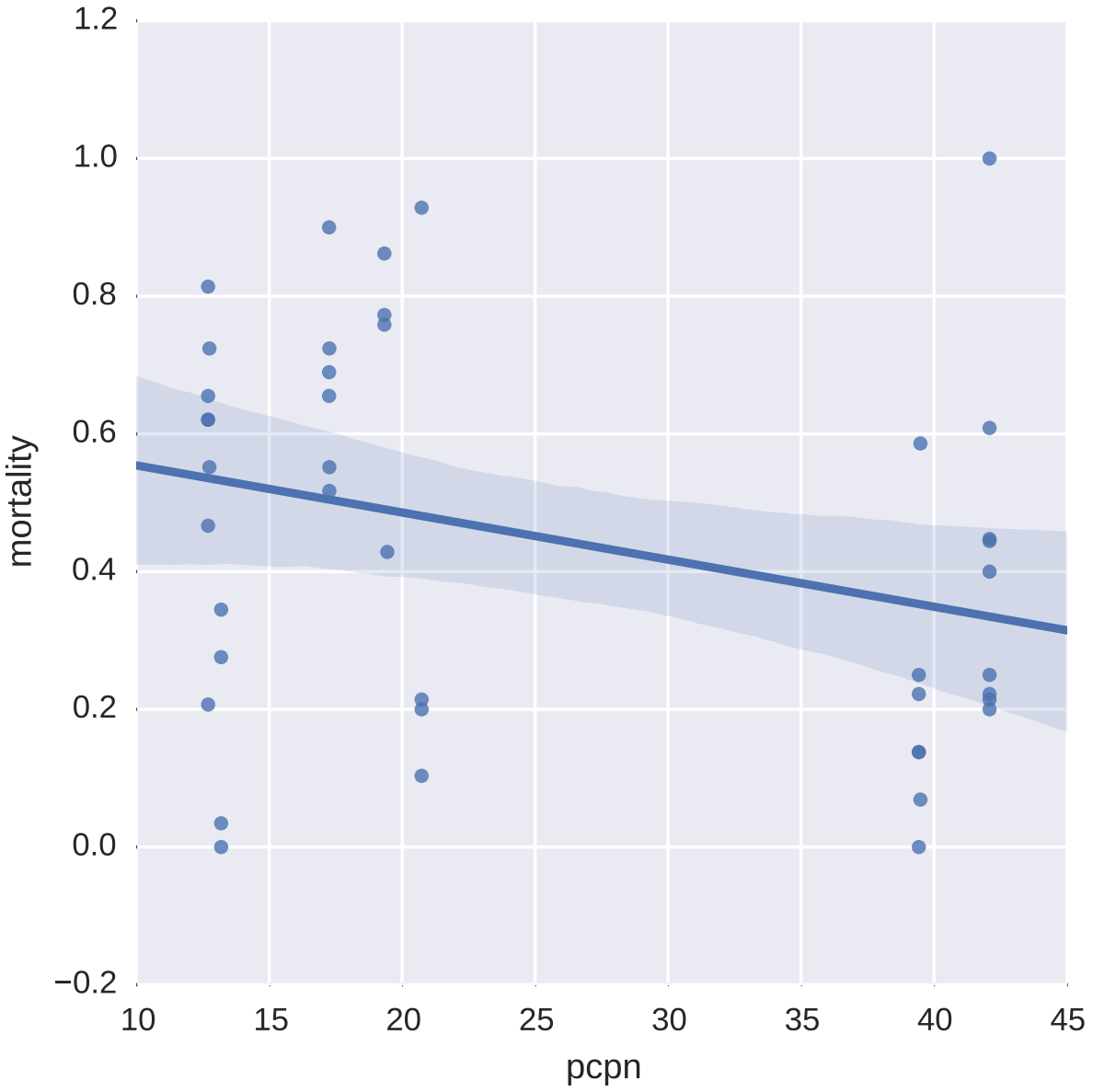

Figure 3: Correlation between mortality and accumulated rainfall in inches during the previous 90 days. (Pearson's correlation coefficient = -0.3053; p = 0.0492).

Appendix 2 - 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.

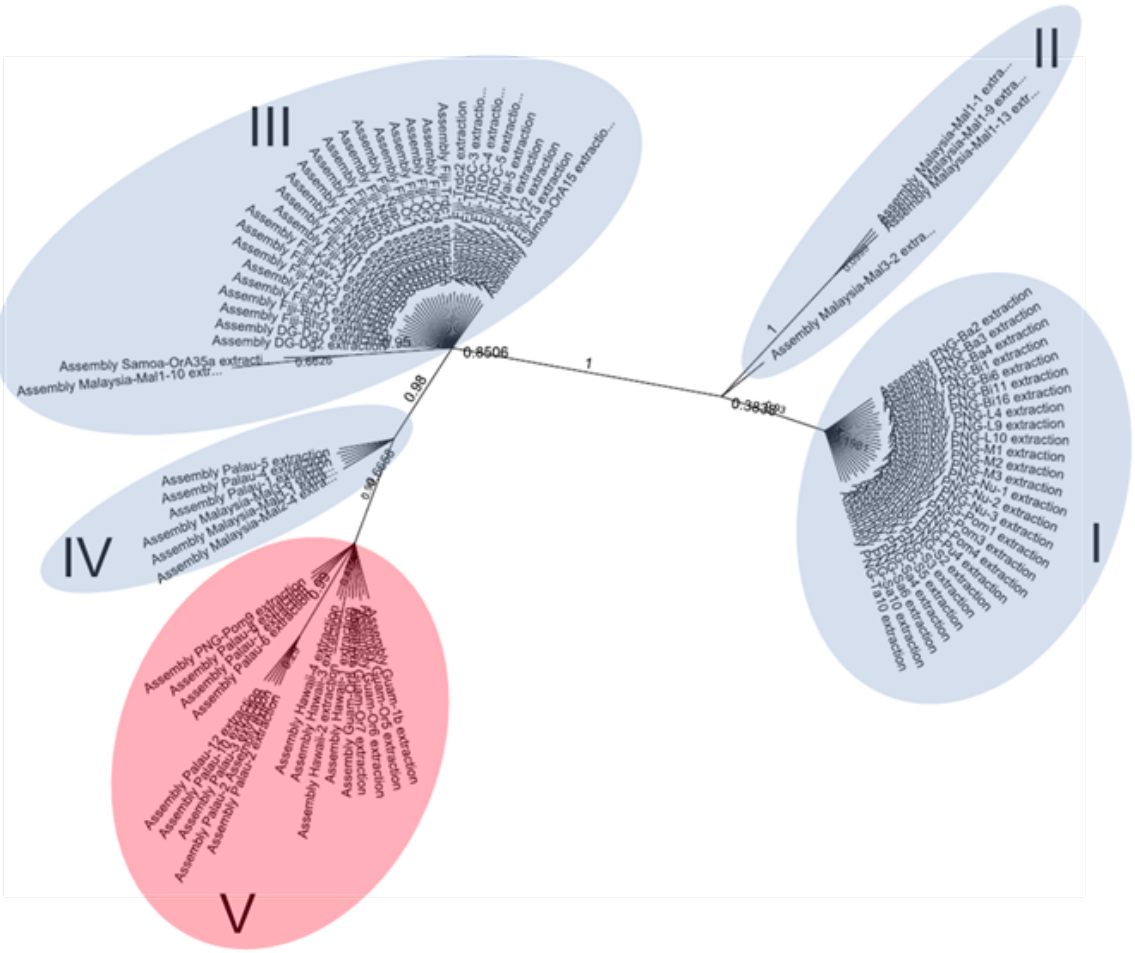

Figure 1: Preliminary phylogenetic analysis of CRB populations based on the COI bar code region. Putative clades are indicated via roman numeral labels plus shading. Clades I to IV represent COI sequence of specimens from Fiji (I), Malaysia (II, IV), PNG (III, V), Palau (IV, V), Hawaii (V), and Guam (V). Members of Clade V all correlate with highly damaging CRB populations and all are positive for the CRB-G haplotype. The majority rule consensus tree includes COI sequences from 95 CRB specimens. Following DNA alignment using Muscle, the constructed tree was inferred from MrBaye phylogenetic analysis Posterior probabilities (from 1.1 x 10^6 generations) are shown at branch nodes. The above software was implemented within the Geneious 8 (BioMatters Ltd) using default settings.

Appendix 3 - Oryctes Nudivirus Witches Brew Experiment

Guam Coconut Rhinoceros Project Technical Report
DRAFT (Experiment Still in Progress)

OrNV Witches Brew Experiment: A Last Ditch Attempt to Find Virus Pathogenetic for the Guam Cocoonut Rhinoceros Beetle Genotype

Aubrey Moore, Ian Iriarte and Roland Quitugua

May 1, 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 May 10, 2015, mortality of the virus treated beetles (78%) was significantly greater than that of the control group (54%).

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'). Significance of differences in mortality were determined using a Fisher's exact test.

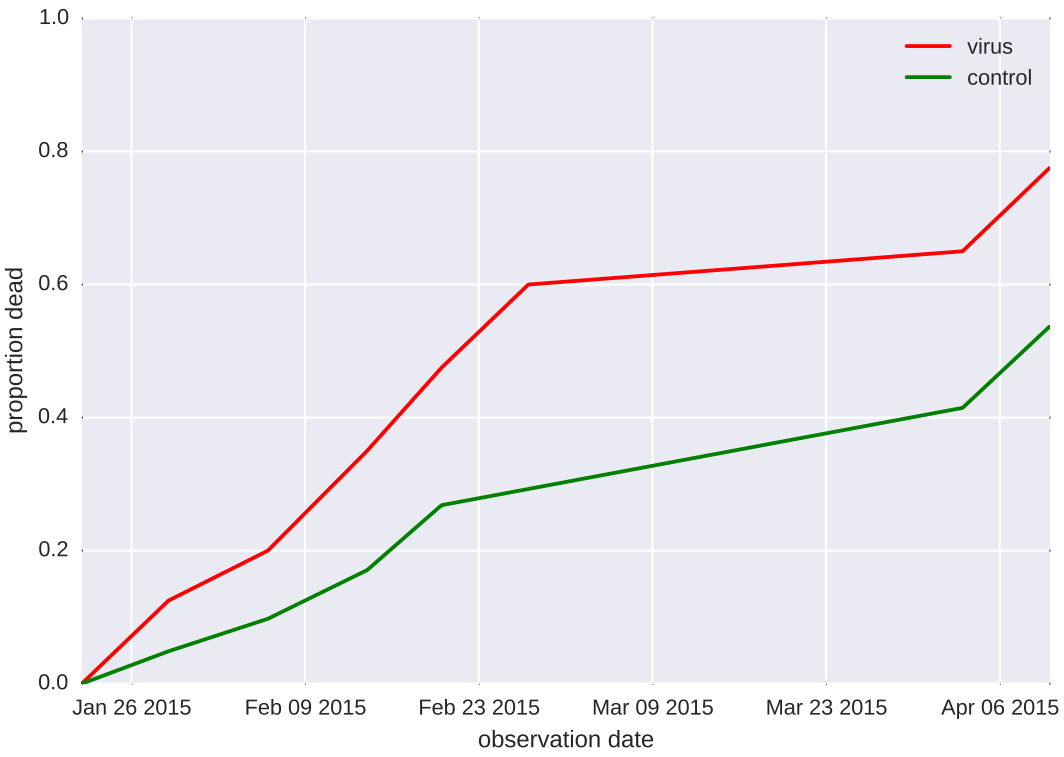

Figure 1: Cumulative mortality.

Results and Discussion

Cumulative mortality of virus-treated beetles (57%) on April 10 (Fig. 1) was significantly greater than that of control beetles (44%); ($p = 0.0005$; Fisher's exact test).

It is unlikely that the beetles which died during the first two weeks of the experiment resulted from exposure to virus, so we removed these from the experimental data and repeated the significance test. This adjustment did not alter the outcome: Mortality of virus-treated beetles (57%) was significantly greater than that of control beetles (44%); ($p = 0.0005$; Fisher's exact test).

This experiment is incomplete. A postmortem will be done on the dead beetles and the 'witches brew' process will be repeated to see if this also results in significant mortality.