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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