CRB haplotype and PCR detection of *Oryctes* nudivirus from field collected *Oryctes rhinoceros* (Guam)

Sean Marshall, Nicky Richards, Caitlin Hyde
June 2021

Report for University of Guam
CLIENT REPORT NUMBER: TBD

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1. EXECUTIVE SUMMARY

- Through a collaboration with the University of Guam, AgResearch (AgR) has analyzed coconut rhinoceros beetle (CRB) specimens sourced from Guam to re-confirm haplotype status of Guam's CRB haplotype status and determine if an Oryctes nudivirus (OrNV isolate may be circulating within this field population.

- This current work has been undertaken as a result of an unexpected OrNV positive result. OrNV was tentatively detected in CRB collected in pheromone traps at Leo Palace and the Yigo Agricultural Experiment Station (YAES).

- A total of 208 CRB specimens (103 from Leo Palace, 105 from YAES) sourced from Guam were analysed.

- All 208 specimens field collected from Guam were of the CRB-G haplotype. These results suggest that the Guam CRB population still corresponds to the CRB-G haplotype based on the PCR-RFLP, which was previously associated with the original initial CRB invasion into Guam.

- A subset of 30 specimens were DNA sequenced across a portion of the COI gene. Sequence analysis of all 30 were identical to COI sequences for specimens associated with the original initial CRB invasion into Guam (i.e. CRB-G Clade I as previously reported).

- None of the 104 specimens field collected from Guam were positive for the presence of OrNV, which corresponds to analyses on Guam sourced CRB from previous reports.

- The results from the field collected CRB specimens provided for this work suggest that no additional CRB haplotypes have entered into Guam since the original invasion was detected in 2007. Additionally, there was no evidence for OrNV being present or circulating within the Guam CRB field population. The most likely explanation for the tentative detection of OrNV is that this resulted from lab contaminated samples; experiments using OrNV were being carried out at the time of the PCR positive OrNV observations.

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2. BACKGROUND

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 (Huger, 1966) 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). Genetic evidence has shown that all these outbreaks were associated with a previously unrecognized haplotype, CRB-G, which appears to be tolerant to OrNV.

The aim of this current study was to confirm the possible presence of OrNV circulating within the Guam CRB population. This has arisen based on CRB specimens collected from two sites in Guam (Leo Palace and the Yigo Agricultural Experiment Station; YAES) where OrNV was tentatively detected in CRB collected from pheromone traps.

3. METHODS

3.1 Collection of *O. rhinoceros* from Guam and genomic DNA extraction from *O. rhinoceros* gut tissue

CRB specimens were collected from netting-based 'Defence' traps deployed at Leo Palace and at the Yigo Agricultural Experiment Station. Traps were baited with ChemTica oryctalure and visited every 2 or 3 days with live individual caught beetles placed in a new sample jar (pottle). The netting-based 'Defence' traps kept individual beetles separate on the trap itself which served to minimize the potential for cross-contamination / cross-infection during trapping should any virus be present in the Guam CRB population. Data recorded for each individual specimen included trap number, trap gps location, number of CRB caught, number taken for tissue sampling.

CRB specimens were sent to AgResearch (New Zealand) for further analysis. The diagnostic methods used for this work were based on those previously described in Marshall et al. 2017.

DNA was extracted from the gut tissue supplied using the Tissue Genomic DNA Mini Kit (Geneaid) column system following manufacturer instructions. DNA elution was carried out using 100 μl of elution buffer and aliquots of eluted DNA samples were subsequently used for further analyses.

3.2 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 *O. rhinoceros COI* gene: C1-J-1718Oryctes (5'- GGAGGTTTCGGAAATTGACTTGTTCC -3') and C1-N-2191Oryctes (5'- CCAGGTAGAATTAAAATRTATACCTC -3'). A unique Tru1I restriction site polymorphism within this amplified region allows the CRB-G haplotype to be identified. Note that Tru1I is an isoschizomer of the MseI restriction enzyme reported in Marshall et al. 2017, and therefore recognizes the same DNA site. Each 20 μl PCR reaction contained: 10 μl VitaTaq® 2X HS Mastermix Gold (Procomcure), 0.4 μl C1-J-1718Oryctes (10 μM), 0.4 μl C1-N-2191Oryctes (10 μM), 1.5 μl 100-fold diluted *O. rhinoceros* DNA template, and 7.7 μl sterile distilled water. PCR amplifications were performed in an Eppendorf Mastercycler Gradient with a cycling profile of 30 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%, 0.5X TBE) alongside a 100 bp DNA Ladder (GeneRuler™, Thermo Scientific). For RFLP analysis, successfully amplified *COI* PCR products (5 µl) were each combined with 0.25 µl Tru1I (10U/µl; Thermo Fisher Scientific), 1.5 µl 10x Buffer R, and 8.25 µl sterile distilled water, and incubated at 65°C for 1 h. The digested samples (15 µl) still contained enough loading dye from the VitaTaq® 2X HS Mastermix Gold to be directly loaded on a 2% agarose gel in 0.5X TBE buffer. RedSafe (iNtRON Biotechnology) was included in all agarose gels and allowed DNA fluorescence to be visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech).

3.3 DNA sequencing of the mitochondrial COI barcode region

For a subset of CRB specimens, 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 Prime software package (Kearse et al., 2012) for further sequence manipulation and analyses to look for differences by comparison with previously reported CRB sequences (see Marshall et al. 2017).

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

O. rhinoceros gut tissue dissected from moribund or dead specimens had DNA extracted as described above. The PCR protocol for detection of OrNV was based on that described in Richards et al. (1999), and has been subsequently modified by using diluted DNA template (down to 1 in 5,000) to better distinguish infection from mere presence due to dosing with OrNV for the pathogen challenge assay. The primer pairs used to amplify a 945 base pair (bp) fragment of the OrNV genome were OrNV15a (5'-ATTACGTCGTAGAGGCAATC-3') and OrNV15b (5'-ATGATCGATTCGTCTATGG-3') (Richards et al., 1999). Each 20 µl PCR reaction contained: 10 µl VitaTaq® 2X HS Mastermix Gold (Procomcure), 0.4 µl OrNV15a (10 µM), 0.4 µl OrNV15b (10 µM), 1.5 µl of the 100-fold or 5,000-fold diluted DNA and 7.7 µl sterile distilled water. PCR amplifications were performed in an Eppendorf Mastercycler Gradient with a cycling profile of 30 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 10 µl aliquot of each PCR reaction was checked by agarose gel electrophoresis (1%, 0.5X TBE) alongside a 100 bp DNA Ladder (GeneRuler™, Thermo Scientific). RedSafe (iNtRON Biotechnology) was included in all agarose gels and allowed DNA fluorescence to be visualized over UV light. Photographs were recorded using an UVIdoc HD2 gel doc (UVItech). Detection of OrNV PCR product in the 1 in 5,000 dilution was considered here as indicative of OrNV infection.

3.5 Histological observation to detect symptoms of *O. rhinoceros* nudivirus infection in dissected *O. rhinoceros* gut tissue

Histological observations of gut samples was carried out for a subset of CRB specimens. Tissue samples are preserved by immersing tissue for 48 h in FAA fixative (5% formaldehyde, 2.5% acetic acid, 50% ethanol as an aqueous solution) before paraffin embedding, serial sectioning, and hematoxylin and eosin (H&E) staining (Kiernan, 1990). Slides of gut tissue were examined under bright-field and differential interference contrast (DIC) optics with observations of OrNV infection status recorded based on pathology described by Huger (2005).

4. RESULTS AND DISCUSSION

4.1 DNA quality and CRB-G haplotype determination

Following genomic DNA extraction of the 208 gut tissue samples received, a test PCR using primers to amplify a 523 bp region the *COI* gene was conducted to confirm the extracted DNA could be PCR amplified. All samples produced the expected partial COI gene product (data not shown), indicating that the extracted DNA was suitable for OrNV analysis in addition to haplotype confirmation.

The RFLP digestion component of the PCR-RFLP haplotype assay was carried out using the above partial *COI* PCR products. A high-l;evel summary of results is presented in Table 1 (see Appendix 1 for individual specimen results), with Figure 1 showing an example anticipated from the assay. All 208 specimens from Guam displayed the digestion pattern expected for CRB-G (bands at 253, 138, 92, 28, 13 base pairs), confirming that the specimens were all CRB-G haplotype.

Table 1: High-level summary of OrNV detection results from *Oryctes rhinoceros* gut tissue received by AgResearch from University of Guam (July and August 2020; specimens collected from Guam).

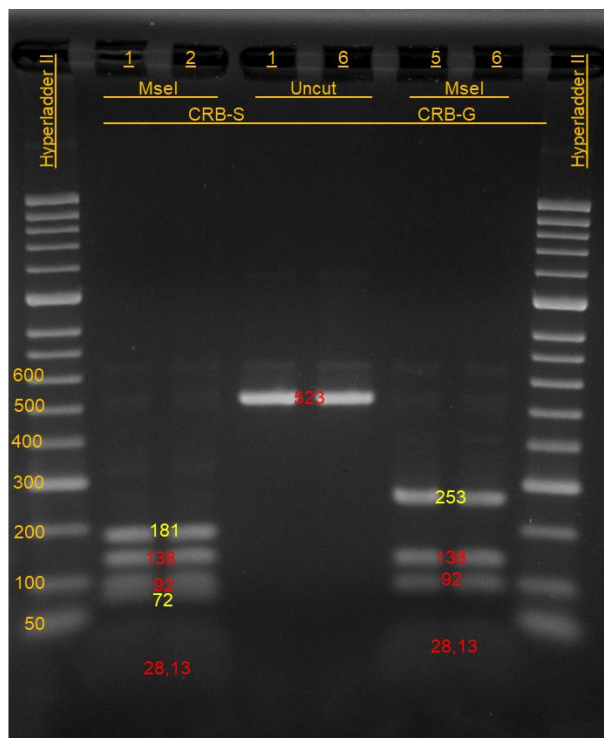| Collection Location	Total # analysed	CRB Biotype	OrNV detected	Comments
Leo. Palace	103	All CRB-G	None	OrNV infection not likely
Yigo Agricultural Experiment Station	105	All CRB-G	None	OrNV infection not likely

Figure 1: Exemplar agarose gel showing PCR-RFLP assay results from *Oryctes rhinoceros* tissue. The pattern of DNA bands seen on the gel indicates the specimens match with the CRB-G (bands at 253, 138, 92, 28, 13 base pairs) or the CRB-S (bands at 181, 138, 92 and 72, 28, and 13 base pairs) haplotype. A 100bp DNA size marker was used with 'ladder rungs' every 100 base pairs as indicated in the figure. Note that MseI and TruI restriction enzymes have the same DNA cleavage site.

4.2 OrNV detection

The dilution PCR analysis of *O. rhinoceros* tissue extracted DNA was used to detect the incidence of OrNV infection from the 208 *O. rhinoceros* gut tissue specimens provided for analysis. A high-l;evel summary of results is presented in Table 1 (see Appendix 1 for individual specimen results). All 208 specimens were negative for OrNV at both dilutions and hence were all scored as OrNV not detected with infection unlikely.

Figure 2 has been included to show exemplar agarose gel PCR assay results for OrNV detection in the '1 in 5,000-diluted' DNA extractions. Please note that the results shown in Figure 2 were not from specimens provide for this study. Presence of a 945 base pair PCR product indicates OrNV DNA is present. Detection of OrNV PCR product in the 1 in 5,000 dilution is considered as indicative of OrNV infection. The marker is in the first lane of each row and has DNA 'ladder rungs' every 100 base pairs, with bolder bands at 500 bp and 1,000 bp. Lanes A, C, and D are examples of PCR positive OrNV results while lanes B, E, F, and G are examples of PCR negative OrNV results.

Histological analysis was attempted on a subsample of 10 tissue samples provided. Unfortunately, as the tissue had been previously frozen, the gut tissue structure was too disrupted to be able to determine the health status. However, Figure 3 has been included as it provides exemplar images of expected results for OrNV infected versus uninfected gut tissue.

Based on the PCR results, no evidence of OrNV infection was detected from any of the 208 CRB gut tissue specimens analyzed from Guam.
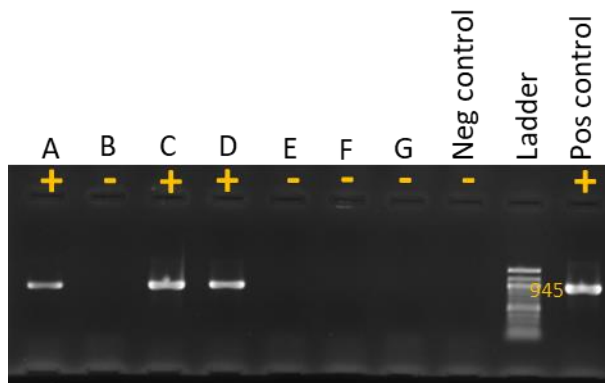

Figure 2: Exemplar agarose gel PCR assay results for OrNV detection in the '1 in 5,000-diluted' DNA extractions from the PNG tissues. The gel for the '1 in 100-diluted DNA' showed identical results and is not shown here. Tissue samples with OrNV detected produce a 945 bp PCR amplicon (+); those without detectable levels of OrNV do not produce a PCR product (-). The marker used has DNA 'ladder rungs' every 100 base pairs, with bolder bands at 500 bp and 1,000 bp.
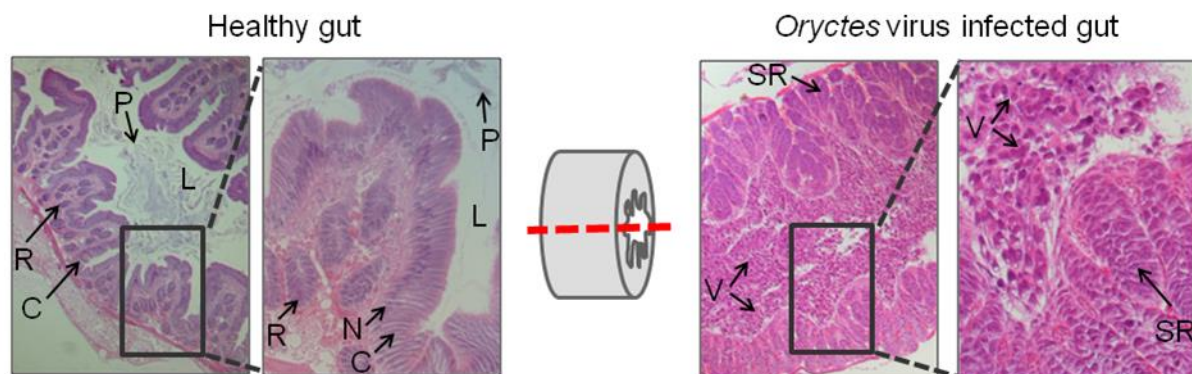

Figure 3: Exemplar images of histological sections highlighting differences observed between healthy and OrNV infected CRB gut tissue. The images of tissue sections show examples of hematoxylin and eosin stained tissue (longitudinal) sections viewed under a light microscope. Compact dark blue/violet staining highlights DNA material (e.g. nucleus), while the cytoplasmic material stains pink with a diffuse blue tint. The central diagram indicates the approximate orientation (red line) of the exemplar tissue sections (grey cylinder). The 'healthy' gut images (left-hand side; 100x and 400x magnification respectively) reveals ordered undulating columns of gut epithelium, with rows of nuclei (N) neatly arranged at the basal end of the columnar cells (C; small dark blue staining). The cells of the regenerative crypts (R) appear as distinct large circular clusters (dark blue stain). The lumen (L) of healthy guts appears free of cell-like vesicles, with portions of peritrophic membrane (P; containing food particles) being generally easily observed. The *Oryctes* nudivirus infected gut images (right-hand side; 100x and 400x magnification respectively) shows that athough the 'waviness' of the gut epithelium can still be observed, the neat columns of columnar cells have disappeared, and the regenerative crypt clusters appear swollen (SR; compare with healthy gut). Additionally, the gut lumen is completely filled with individual round cell-like vesicles (V) that have been sloughed from the gut epithelium; neither 'clear' space nor peritrophic membrane can be observed.

5. CONCLUSIONS and RECOMMENDATIONS

- All 208 of the CRB specimens collected from two separate areas on Guam (Leo Palace and Yigo Agricultural Experimental Station) were of the CRB-G haplotype. The CRB-G haplotype was previously reported as the only one present in Guam, and shown to be tolerant to infection by the OrNV isolates released as biocontrol agents in the Pacific.
- The PCR detection results were negative for all 208 samples at both DNA dilutions, which indicates that OrNV could not be detected and therefore infection was unlikely.
- For future preservation of tissue intended for histological analysis, these should be preserved and stored in a formaldehyde-based preservative (e.g. FAA as described in methods section, or 10% neutral buffered formalin), with preserved specimens maintained at ambient room temperature (~20-30°C). Freezing or replacement of the formaldehyde-based preservative with DNA preservative solutions (e.g. MPG, ethanol) can both damage tissue and cell structure, which creates artifacts that interfere with examination for diagnosis.
- As no evidence of OrNV presence was found in any of the 208 CRB from Guam, it is highly unlikely for virus to be present or circulating within the field CRB population of Guam. It has been noted that CRB-OrNV challenge bioassays were being carried out in the lab at the time a few Guam sourced CRB-G specimens were tentatively identified as OrNV positive. Therefore, it appears most likely that the virus positive results arose from accidental lab cross-contamination / cross-infection.
- Wherever possible, separation of 'healthly' CRB and work OrNV work in time and/or space is recommended to avoid accidental cross-contamination / cross-infection of virus between insects. If separate rooms are not feasible, closed storage boxes or designated areas for virus only or non-virus only areas may be an option.

6. ACKNOWLEDGEMENTS

7. REFERENCES

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Appendix 1: Summary of individual diagnostic results from *Oryctes rhinoceros* gut tissue received by AgResearch from University of Guam (specimens collected from Guam July and August 2020)

AgR ID	Tube Label	Collection Location	GPS Northing	GPS Easting	Gender	CRB Biotype	OrNV detected	Comments
20-0724	bbd99097	L. Palace, Trap: c700cb2a	13.411634	144.734561	f	CRB-G	No	OrNV infection not likely
20-0725	f7ef03b5	L. Palace, Trap: 79936575	13.410638	144.736089	f	CRB-G	No	OrNV infection not likely
20-0726	bef5c2db	L. Palace, Trap: c700cb2a	13.411634	144.734561	m	CRB-G	No	OrNV infection not likely
20-0727	479c48e3	L. Palace, Trap: 91bc5abc	13.406979	144.740393	f	CRB-G	No	OrNV infection not likely
20-0728	80a32fb2	L. Palace, Trap: 2849ea04	13.408627	144.740062	f	CRB-G	No	OrNV infection not likely
20-0729	dd1d644c	L. Palace, Trap: 16d0f9c5	13.414568	144.732996	f	CRB-G	No	OrNV infection not likely
20-0730	28cc0d03	L. Palace, Trap: 79936575	13.410638	144.736089	f	CRB-G	No	OrNV infection not likely
20-0731	a168bf88	L. Palace, Trap: 79936575	13.410638	144.736089	f	CRB-G	No	OrNV infection not likely
20-0732	e9e996fb	L. Palace, Trap: f5c4388e	13.414025	144.733288	f	CRB-G	No	OrNV infection not likely
20-0733	8d471434	L. Palace, Trap: 09c078e4	13.412643	144.733926	m	CRB-G	No	OrNV infection not likely
20-0734	5c57da4a	L. Palace, Trap: f53ef5cf	13.413028	144.733589	m	CRB-G	No	OrNV infection not likely
20-0735	be9ffad0	L. Palace, Trap: 79936575	13.410638	144.736089	f	CRB-G	No	OrNV infection not likely
20-0736	17c37b97	L. Palace, Trap: f53ef5cf	13.413028	144.733589	m	CRB-G	No	OrNV infection not likely
20-0737	926b60f1	L. Palace, Trap: 05e90a7c	13.411265	144.7355097	m	CRB-G	No	OrNV infection not likely
20-0738	7f60ec05p-tube (7f60ec05-paperwork)	L. Palace, Trap: 05e90a7c	13.411265	144.7355097	m	CRB-G	No	OrNV infection not likely
20-0739	57bf436c	L. Palace, Trap: 22d96197	13.408251	144.740266	m	CRB-G	No	OrNV infection not likely
20-0740	3f56899f	L. Palace, Trap: a21e4bc2	13.410892	144.735548	f	CRB-G	No	OrNV infection not likely
20-0741	b1148c85	L. Palace, Trap: bc825819	13.407736	144.740402	m	CRB-G	No	OrNV infection not likely
20-0742	1b24ea86-tube (1b24e986-paperwork)	L. Palace, Trap: f5c4388e	13.414025	144.733288	f	CRB-G	No	OrNV infection not likely

20-0743	89b53249	L. Palace, Trap: 2f303657	13.411874	144.74066	m	CRB-G	No	OrNV infection not likely
20-0744	3e0d62f4	L. Palace, Trap: 6a7c6569	13.405281	144.740517	m	CRB-G	No	OrNV infection not likely
20-0745	a479f23c	L. Palace, Trap: d68893d0	13.41358	144.733495	f	CRB-G	No	OrNV infection not likely
20-0746	9252f2cf	L. Palace, Trap: 6a7c6569	13.405281	144.740517	f	CRB-G	No	OrNV infection not likely
20-0747	05cadfe4	L. Palace, Trap: c700cb2a	13.411634	144.734561	f	CRB-G	No	OrNV infection not likely
20-0748	2b62e865	L. Palace, Trap: 91bc5abc	13.406979	144.740393	m	CRB-G	No	OrNV infection not likely
20-0749	e645b55c	L. Palace, Trap: 6ba2354e	13.405281	144.740789	m	CRB-G	No	OrNV infection not likely
20-0750	283c3840	L. Palace, Trap: 16d0f9c5	13.414568	144.732996	f	CRB-G	No	OrNV infection not likely
20-0751	f2e172f2	L. Palace, Trap: 16d0f9c5	13.414568	144.732996	m	CRB-G	No	OrNV infection not likely
20-0752	51590808	L. Palace, Trap: aec1146a	13.413888	144.738991	m	CRB-G	No	OrNV infection not likely
20-0753	c93ffed4	L. Palace, Trap: 22d96197	13.408251	144.740266	m	CRB-G	No	OrNV infection not likely
20-0754	fba25f39	L. Palace, Trap: 22d96197	not recorded		f	CRB-G	No	OrNV infection not likely
20-0755	42e419f9	L. Palace, Trap: d56202e4	13.404666	144.740866	f	CRB-G	No	OrNV infection not likely
20-0756	fff691fd	L. Palace, Trap: f7209075	13.412871	144.739543	m	CRB-G	No	OrNV infection not likely
20-0757	b5ee3c2e	L. Palace, Trap: 79936575	13.410638	144.736089	m	CRB-G	No	OrNV infection not likely
20-0758	61dfedaa	L. Palace, Trap: 0b95fdc1	13.414568	144.732996	f	CRB-G	No	OrNV infection not likely
20-0759	a6473274	L. Palace, Trap: 9c04998a	13.413562	144.739315	f	CRB-G	No	OrNV infection not likely
20-0760	0aab246d	L. Palace, Trap: 0b95fdc1	13.414568	144.732996	m	CRB-G	No	OrNV infection not likely
20-0761	e429bf01	L. Palace, Trap: 7e396304	13.416147	144.742125	f	CRB-G	No	OrNV infection not likely
20-0762	c46442d0	L. Palace, Trap: f80ed757	13.415421	144.742386	m	CRB-G	No	OrNV infection not likely
20-0763	cdfb1bfe	L. Palace, Trap: aec1146a	13.413888	144.738991	f	CRB-G	No	OrNV infection not likely
20-0764	4d5250c4	L. Palace, Trap: f469d36a	13.412352	144.740216	f	CRB-G	No	OrNV infection not likely
20-0765	b0291e7a	L. Palace, Trap: not recorded	not recorded		m	CRB-G	No	OrNV infection not likely

20-0766	45b87a23	L. Palace, Trap: d56202e4	13.404666	144.740866	m	CRB-G	No	OrNV infection not likely
20-0767	de99cded	L. Palace, Trap: 3abc4df2	13.411268	144.741565	f	CRB-G	No	OrNV infection not likely
20-0768	08acebf8	L. Palace, Trap: 0b95fdc1	13.414568	144.732996	m	CRB-G	No	OrNV infection not likely
20-0769	f36c857d	L. Palace, Trap: d56202e4	13.404666	144.740866	f	CRB-G	No	OrNV infection not likely
20-0770	efdd0aa2	L. Palace, Trap: 0b95fdc1	13.414568	144.732996	f	CRB-G	No	OrNV infection not likely
20-0771	d2086acb	L. Palace, Trap: aec1146a	13.413888	144.738991	f	CRB-G	No	OrNV infection not likely
20-0772	a2c841f2	L. Palace, Trap: d68893d0	13.41358	144.733495	m	CRB-G	No	OrNV infection not likely
20-0773	3ba1b335	L. Palace, Trap: f7209075	13.412871	144.739543	m	CRB-G	No	OrNV infection not likely
20-0774	c923e103	L. Palace, Trap: 9c04998a	13.413562	144.739315	f	CRB-G	No	OrNV infection not likely
20-0775	d0e5dfbd	L. Palace, Trap: f5c4388e	13.414025	144.733288	m	CRB-G	No	OrNV infection not likely
20-0776	e42f0aa6	L. Palace, Trap: 01c1beea	13.416949	144.745809	m	CRB-G	No	OrNV infection not likely
20-0777	ce617e04	L. Palace, Trap: 9c04998a	13.413562	144.739315	m	CRB-G	No	OrNV infection not likely
20-0778	2e4b2282	L. Palace, Trap: f80ed757	13.415421	144.742386	f	CRB-G	No	OrNV infection not likely
20-0779	fe75b1aa	L. Palace, Trap: f469d36a	13.412352	144.740216	m	CRB-G	No	OrNV infection not likely
20-0780	1a9f26b9	L. Palace, Trap: 9c04998a	13.413562	144.739315	f	CRB-G	No	OrNV infection not likely
20-0781	b442905e	L. Palace, Trap: bc825819	13.407736	144.740402	m	CRB-G	No	OrNV infection not likely
20-0782	66b7662c	L. Palace, Trap: 01c1beea	13.416949	144.745809	m	CRB-G	No	OrNV infection not likely
20-0783	20a3d720	L. Palace, Trap: 3c1dd8db	13.417106	144.742955	f	CRB-G	No	OrNV infection not likely
20-0784	d6e081f2	L. Palace, Trap: f5c4388e	13.414025	144.733288	m	CRB-G	No	OrNV infection not likely
20-0785	17be3474	L. Palace, Trap: d56202e4	13.404666	144.740866	f	CRB-G	No	OrNV infection not likely
20-0786	70e746cc	L. Palace, Trap: b76fc66d	13.416766	144.742557	f	CRB-G	No	OrNV infection not likely
20-0787	d1ab8a8e	L. Palace, Trap: f80ed757	13.415421	144.742386	m	CRB-G	No	OrNV infection not likely
20-0788	615c7d53	L. Palace, Trap: 4ac78b16	13.417579	144.74404	f	CRB-G	No	OrNV infection not likely

20-0789	972ba48a	L. Palace, Trap: f80ed757	13.415421	144.742386	m	CRB-G	No	OrNV infection not likely
20-0790	867d5c63	L. Palace, Trap: f0d8f2ae	13.41795	144.743813	m	CRB-G	No	OrNV infection not likely
20-0791	095dbca3	L. Palace, Trap: f0d8f2ae	13.41795	144.743813	f	CRB-G	No	OrNV infection not likely
20-0792	5a196c73	L. Palace, Trap: 0b95fdc1	13.414568	144.732996	m	CRB-G	No	OrNV infection not likely
20-0793	6d675659	L. Palace, Trap: f0d8f2ae	13.41795	144.743813	f	CRB-G	No	OrNV infection not likely
20-0794	8e0c1574	L. Palace, Trap: 01c1beea	13.416949	144.745809	m	CRB-G	No	OrNV infection not likely
20-0795	c13124cc	L. Palace, Trap: f80ed757	13.415421	144.742386	m	CRB-G	No	OrNV infection not likely
20-0796	ae2d739f	L. Palace, Trap: aec1146a	13.413888	144.738991	f	CRB-G	No	OrNV infection not likely
20-0797	c20c9557	L. Palace, Trap: 7e396304	13.416147	144.742125	f	CRB-G	No	OrNV infection not likely
20-0798	d3ad1cf9	L. Palace, Trap: f80ed757	13.415421	144.742386	f	CRB-G	No	OrNV infection not likely
20-0799	e7cc6f83	L. Palace, Trap: cf674b41	13.409112	144.737028	m	CRB-G	No	OrNV infection not likely
20-0800	f023ba62	L. Palace, Trap: dFd'c3de	13.406569	144.74074	f	CRB-G	No	OrNV infection not likely
20-0801	focd421d	L. Palace, Trap: 7e396304	13.416147	144.742125	m	CRB-G	No	OrNV infection not likely
20-0802	bc41db0a	L. Palace, Trap: f0d8f2ae	13.41795	144.743813	m	CRB-G	No	OrNV infection not likely
20-0803	b3d20683	L. Palace, Trap: 64b6d8df	13.412575	144.739812	f	CRB-G	No	OrNV infection not likely
20-0804	ea102604	L. Palace, Trap: 4ac78b16	13.417579	144.74404	m	CRB-G	No	OrNV infection not likely
20-0805	9e0cb7c6	L. Palace, Trap: 01c1beea	13.416949	144.745809	f	CRB-G	No	OrNV infection not likely
20-0806	89ca63f7	L. Palace, Trap: d2a07606	13.417591	144.743189	m	CRB-G	No	OrNV infection not likely
20-0807	a50407d5	L. Palace, Trap: b76fc66d	13.416766	144.742557	m	CRB-G	No	OrNV infection not likely
20-0808	7e230a3e	L. Palace, Trap: f80ed757	13.415421	144.742386	m	CRB-G	No	OrNV infection not likely
20-0809	e56a1b7c	L. Palace, Trap: 2f303657	13.411874	144.74066	f	CRB-G	No	OrNV infection not likely
20-0810	857b9c91	L. Palace, Trap: 3c1dd8db	13.417106	144.742955	m	CRB-G	No	OrNV infection not likely
20-0811	f0faa045	Yigo, Trap: e4232f69	13.529843	144.874082	f	CRB-G	No	OrNV infection not likely

20-0812	87464f40	L. Palace, Trap: e7d5ea5d	13.417849	144.744333	f	CRB-G	No	OrNV infection not likely
20-0813	80535f0d	L. Palace, Trap: 22d96197	13.408251	144.740266	f	CRB-G	No	OrNV infection not likely
20-0814	0a07fe75	L. Palace, Trap: e7d5ea5d	13.417849	144.744333	m	CRB-G	No	OrNV infection not likely
20-0815	c15a1212	L. Palace, Trap: e7d5ea5d	13.417849	144.744333	f	CRB-G	No	OrNV infection not likely
20-0816	e5a83bc8	L. Palace, Trap: cf674b41	13.409112	144.737028	f	CRB-G	No	OrNV infection not likely
20-0817	80a09b35	L. Palace, Trap: cf674b41	13.409112	144.737028	f	CRB-G	No	OrNV infection not likely
20-0818	b41124ea	L. Palace, Trap: cf674b41	13.409112	144.737028	f	CRB-G	No	OrNV infection not likely
20-0819	d0841ffe	L. Palace, Trap: e7d5ea5d	13.417849	144.744333	f	CRB-G	No	OrNV infection not likely
20-0820	6d9b0a11	L. Palace, Trap: 4ac78b16	13.417579	144.74404	f	CRB-G	No	OrNV infection not likely
20-0821	dba15da6	L. Palace, Trap: cf674b41	13.409112	144.737028	m	CRB-G	No	OrNV infection not likely
20-0822	c98a0534	L. Palace, Trap: 01c1beea	13.416949	144.745809	m	CRB-G	No	OrNV infection not likely
20-0823	4c4803c0	L. Palace, Trap: 01c1beea	13.416949	144.745809	f	CRB-G	No	OrNV infection not likely
20-0824	443a949e	L. Palace, Trap: 01c1beea	13.416949	144.745809	m	CRB-G	No	OrNV infection not likely
20-0825	136545e2-tube (1.37E+07-paperwork)	L. Palace, Trap: e7d5ea5d	13.417849	144.744333	m	CRB-G	No	OrNV infection not likely
20-0826	e19472f3	L. Palace, Trap: not recorded	13.417849	144.744333	not recorded	CRB-G	No	OrNV infection not likely
20-0827	8eb5d61a	L. Palace, Trap: not recorded	13.413028	144.733589	not recorded	CRB-G	No	OrNV infection not likely
20-1020	4909e798	Yigo, Trap: 3d073d05	13.523428	144.860574	m	CRB-G	No	OrNV infection not likely
20-1021	c25c9e96	Yigo, Trap: 3d073d05	13.523428	144.860574	f	CRB-G	No	OrNV infection not likely
20-1022	8f0c5340	Yigo, Trap: ee9dabe3	13.533097	144.872441	f	CRB-G	No	OrNV infection not likely
20-1023	f202d529	Yigo, Trap: 87a9df43	13.533565	144.874834	f	CRB-G	No	OrNV infection not likely
20-1024	785e3979	Yigo, Trap: 3d073d05	13.523428	144.860574	m	CRB-G	No	OrNV infection not likely
20-1025	e99fd519	Yigo, Trap: ee9dabe3	13.533097	144.872441	m	CRB-G	No	OrNV infection not likely
20-1026	3218a6d7	Yigo, Trap: 247095d5	13.53326	144.869856	m	CRB-G	No	OrNV infection not likely
20-1027	a5504330	Yigo, Trap: e4232f69	13.529843	144.874082	f	CRB-G	No	OrNV infection not likely
20-1028	fd9b9a80	Yigo, Trap: e4232f69	13.529843	144.874082	f	CRB-G	No	OrNV infection not likely
20-1029	8ddf53e1	Yigo, Trap: 3d073d05	13.523428	144.860574	m	CRB-G	No	OrNV infection not likely

20-1030	b764691e	Yigo, Trap: 2662de7f	13.522042	144.871231	f	CRB-G	No	OrNV infection not likely
20-1031	95968fd6	Yigo, Trap: 089d0541	13.529708	144.87218	m	CRB-G	No	OrNV infection not likely
20-1032	3f439c2b	Yigo, Trap: 089d0541	13.529708	144.87218	f	CRB-G	No	OrNV infection not likely
20-1033	92237c37	Yigo, Trap: e4232f69	13.529843	144.874082	f	CRB-G	No	OrNV infection not likely
20-1034	0899a2d4	Yigo, Trap: ee9dabe3	13.533097	144.872441	f	CRB-G	No	OrNV infection not likely
20-1035	ce08a0c0	Yigo, Trap: 7f3dfd7c	13.528865	144.871663	m	CRB-G	No	OrNV infection not likely
20-1036	17bc38ba	Yigo, Trap: ee9dabe3	13.533097	144.872441	m	CRB-G	No	OrNV infection not likely
20-1037	dce4b362	Yigo, Trap: 4784f074	13.532693	144.87188	m	CRB-G	No	OrNV infection not likely
20-1038	592f69db	Yigo, Trap: ee9dabe3	13.533097	144.872441	m	CRB-G	No	OrNV infection not likely
20-1039	45cc2074	Yigo, Trap: ee9dabe3	13.533097	144.872441	m	CRB-G	No	OrNV infection not likely
20-1040	8ac5be95	Yigo, Trap: not recorded	not recorded		f	CRB-G	No	OrNV infection not likely
20-1041	6dfc4537	Yigo, Trap: 7f3dfd7c	13.528865	144.871663	m	CRB-G	No	OrNV infection not likely
20-1042	5da246d9	Yigo, Trap: 089d0541	13.529708	144.87218	f	CRB-G	No	OrNV infection not likely
20-1043	607dbd9e	Yigo, Trap: 3d073d05	13.523428	144.860574	f	CRB-G	No	OrNV infection not likely
20-1044	8f9a8029	Yigo, Trap: 089d0541	13.529708	144.87218	m	CRB-G	No	OrNV infection not likely
20-1045	0dc10361	Yigo, Trap: 089d0541	13.529708	144.87218	m	CRB-G	No	OrNV infection not likely
20-1046	ecf672e9	Yigo, Trap: ee9dabe3	13.533097	144.872441	f	CRB-G	No	OrNV infection not likely
20-1047	3bf99884	Yigo, Trap: 3d073d05	13.523428	144.860574	f	CRB-G	No	OrNV infection not likely
20-1048	cb5c8125	Yigo, Trap: 089d0541	13.529708	144.87218	f	CRB-G	No	OrNV infection not likely
20-1049	2de6091d	Yigo, Trap: 4784f074	13.532693	144.87188	f	CRB-G	No	OrNV infection not likely
20-1050	6c30c880	Yigo, Trap: ee9dabe3	13.533097	144.872441	f	CRB-G	No	OrNV infection not likely
20-1051	3796c8bf	Yigo, Trap: 2b2de423	13.530552	144.874284	m	CRB-G	No	OrNV infection not likely
20-1052	7e29455c	Yigo, Trap: ee9dabe3	13.533097	144.872441	f	CRB-G	No	OrNV infection not likely
20-1053	fc74e586	Yigo, Trap: ee9dabe3	13.533097	144.872441	m	CRB-G	No	OrNV infection not likely
20-1054	520a5b0b	Yigo, Trap: ee9dabe3	13.533097	144.872441	m	CRB-G	No	OrNV infection not likely
20-1055	bc87655a	Yigo, Trap: ee9dabe3	13.533097	144.872441	m	CRB-G	No	OrNV infection not likely
20-1056	199072ac	Yigo, Trap: 247095d5	13.53326	144.869856	m	CRB-G	No	OrNV infection not likely
20-1057	24aea1a0	Yigo, Trap: 247095d5	13.53326	144.869856	f	CRB-G	No	OrNV infection not likely
20-1058	4eefcda2	Yigo, Trap: 247095d5	13.53326	144.869856	f	CRB-G	No	OrNV infection not likely
20-1059	8cc0d1a5	Yigo, Trap: ba460c56	13.532277	144.871768	f	CRB-G	No	OrNV infection not likely
20-1060	a0346aa0	Yigo, Trap: ad9d1802	13.532889	144.874734	m	CRB-G	No	OrNV infection not likely

20-1061	66dc8ac1	Yigo, Trap: not recorded	not recorded		f	CRB-G	No	OrNV infection not likely
20-1062	bdbc8650	Yigo, Trap: not recorded	not recorded		f	CRB-G	No	OrNV infection not likely
20-1063	cf611e87	Yigo, Trap: ba460c56	13.532277	144.871768	m	CRB-G	No	OrNV infection not likely
20-1064	ca16ae00	Yigo, Trap: 89c246cf	13.531152	144.87117	f	CRB-G	No	OrNV infection not likely
20-1065	d9276fcb	Yigo, Trap: 3d073d05	13.523428	144.860574	f	CRB-G	No	OrNV infection not likely
20-1066	8135cbdf	Yigo, Trap: 4784f074	13.532693	144.87188	f	CRB-G	No	OrNV infection not likely
20-1067	50da5554	Yigo, Trap: ba460c56	13.532277	144.871768	f	CRB-G	No	OrNV infection not likely
20-1068	5c355642	Yigo, Trap: ba460c56	13.532277	144.871768	f	CRB-G	No	OrNV infection not likely
20-1069	ff898aaf	Yigo, Trap: 47328a7b	13.530849	144.874681	m	CRB-G	No	OrNV infection not likely
20-1070	3bf1e5e3	Yigo, Trap: ba460c56	13.532277	144.871768	m	CRB-G	No	OrNV infection not likely
20-1071	19efff6d	Yigo, Trap: 7f3dfd7c	13.528865	144.871663	f	CRB-G	No	OrNV infection not likely
20-1072	ef4ad0e5	Yigo, Trap: 4784f074	13.532693	144.87188	m	CRB-G	No	OrNV infection not likely
20-1073	fd6e6c32	Yigo, Trap: 7f3dfd7c	13.528865	144.871663	m	CRB-G	No	OrNV infection not likely
20-1074	fdbfcbaf	Yigo, Trap: d043bc35	13.532169	144.871211	f	CRB-G	No	OrNV infection not likely
20-1075	cf836708	Yigo, Trap: 2b2de423	13.530552	144.874284	f	CRB-G	No	OrNV infection not likely
20-1076	65cd68cb	Yigo, Trap: 3d073d05	13.523428	144.860574	m	CRB-G	No	OrNV infection not likely
20-1077	0e6b7e0a	Yigo, Trap: d8f11add	13.532105	144.87164	f	CRB-G	No	OrNV infection not likely
20-1078	8c68571c	Yigo, Trap: 2b2de423	13.530552	144.874284	f	CRB-G	No	OrNV infection not likely

20-1079	17a28b0f	Yigo, Trap: 555803ce	13.534916	144.870543	m	CRB-G	No	OrNV infection not likely
20-1080	c5887164	Yigo, Trap: 2662de7f	13.522042	144.871231	f	CRB-G	No	OrNV infection not likely
20-1081	f2ac9c31	Yigo, Trap: 2022d4ec	13.53197	144.872361	m	CRB-G	No	OrNV infection not likely
20-1082	c744cb6b	Yigo, Trap: ac646324	13.532678	144.871081	f	CRB-G	No	OrNV infection not likely
20-1083	1c498d51	Yigo, Trap: ad9d1802	13.532889	144.874734	f	CRB-G	No	OrNV infection not likely
20-1084	f4e4f1d3	Yigo, Trap: 80bf7a07	13.529743	144.872462	f	CRB-G	No	OrNV infection not likely
20-1085	0753b7bd	Yigo, Trap: 2b2de423	13.530552	144.874284	m	CRB-G	No	OrNV infection not likely
20-1086	1027ed72	Yigo, Trap: 2022d4ec	13.53197	144.872361	f	CRB-G	No	OrNV infection not likely
20-1087	7da3e618	Yigo, Trap: 2b2de423	13.530552	144.874284	f	CRB-G	No	OrNV infection not likely
20-1089	de99cded	Yigo, Trap: 3abc4df2	13.411268	144.741565	f	CRB-G	No	OrNV infection not likely
20-1090	5ffa1623	Yigo, Trap: 247095d5	13.53326	144.869856	m	CRB-G	No	OrNV infection not likely
20-1091	b115513f	Yigo, Trap: not recorded	not recorded		m	CRB-G	No	OrNV infection not likely
20-1092	f805acd6	Yigo, Trap: 2b2de423	13.530552	144.874284	m	CRB-G	No	OrNV infection not likely
20-1093	8b78e8bc	Yigo, Trap: 6821834	13.531344	144.873766	f	CRB-G	No	OrNV infection not likely
20-1094	0736c500	Yigo, Trap: ba460c56	13.532277	144.871768	m	CRB-G	No	OrNV infection not likely
20-1095	d68461c5	Yigo, Trap: 4784f074	13.532693	144.87188	f	CRB-G	No	OrNV infection not likely
20-1096	b47907ca	Yigo, Trap: 7f3dfd7c	13.528865	144.871663	m	CRB-G	No	OrNV infection not likely
20-1097	4b7caa8e	Yigo, Trap: d043bc35	13.532169	144.871211	f	CRB-G	No	OrNV infection not likely

20-1098	87316a48	Yigo, Trap: not recorded	not recorded		m	CRB-G	No	OrNV infection not likely
20-1099	2a7195f8	Yigo, Trap: not recorded	not recorded		m	CRB-G	No	OrNV infection not likely
20-1100	4fc6991e	Yigo, Trap: ad9d1802	13.532889	144.874734	f	CRB-G	No	OrNV infection not likely
20-1101	597bcd3a	Yigo, Trap: 7f3dfd7c	13.528865	144.871663	m	CRB-G	No	OrNV infection not likely
20-1102	68ec88cc	Yigo, Trap: cb45e887	13.530617	144.870651	f	CRB-G	No	OrNV infection not likely
20-1103	7fc96371	Yigo, Trap: not recorded	not recorded		f	CRB-G	No	OrNV infection not likely
20-1104	fb0db17f	Yigo, Trap: cb45e887	13.530617	144.870651	m	CRB-G	No	OrNV infection not likely
20-1105	9d5beb5c	Yigo, Trap: 3d073d05	13.523428	144.860574	m	CRB-G	No	OrNV infection not likely
20-1106	e1002b6b	Yigo, Trap: accb290d	13.533507	144.870805	f	CRB-G	No	OrNV infection not likely
20-1107	1fcf9c78	Yigo, Trap: 247095d5	13.53326	144.869856	f	CRB-G	No	OrNV infection not likely
20-1108	bb2b54ee	Yigo, Trap: 555803ce	13.534916	144.870543	m	CRB-G	No	OrNV infection not likely
20-1109	f1ae3e15	Yigo, Trap: 4784f074	13.532693	144.87188	m	CRB-G	No	OrNV infection not likely
20-1110	d48bb68f	Yigo, Trap: not recorded	not recorded		f	CRB-G	No	OrNV infection not likely
20-1111	8c41c38a	Yigo, Trap: 555803ce	13.534916	144.870543	m	CRB-G	No	OrNV infection not likely
20-1112	78d526ad	Yigo, Trap: 3d073d05	13.523428	144.860574	f	CRB-G	No	OrNV infection not likely
20-1113	8.3846E+14	Yigo, Trap: 247095d5	13.53326	144.869856	m	CRB-G	No	OrNV infection not likely
20-1114	275fb353	Yigo, Trap: 4784f074	13.532693	144.87188	m	CRB-G	No	OrNV infection not likely
20-1115	ba671058	Yigo, Trap: 45591037	13.532029	144.871608	f	CRB-G	No	OrNV infection not likely

20-1116	ba17e4c4	Yigo, Trap: not recorded	not recorded		f	CRB-G	No	OrNV infection not likely
20-1117	40686225	Yigo, Trap: 4784f074	13.532693	144.87188	f	CRB-G	No	OrNV infection not likely
20-1118	e0812a09	Yigo, Trap: d94adb8c	13.531792	144.874233	f	CRB-G	No	OrNV infection not likely
20-1119	837aa8dd	Yigo, Trap: 076d53bc	13.532081	144.873406	f	CRB-G	No	OrNV infection not likely
20-1120	f3441a52	Yigo, Trap: 2b2de423	13.530552	144.874284	m	CRB-G	No	OrNV infection not likely
20-1121	41062a3b	Yigo, Trap: 9c04998a	13.413562	144.739315	not recorded	CRB-G	No	OrNV infection not likely
20-1122	55304d0e	Yigo, Trap: c700cb2a	13.411634	144.734561	not recorded	CRB-G	No	OrNV infection not likely
20-1123	654e0889	Yigo, Trap: 3d073d05	13.523428	144.860574	not recorded	CRB-G	No	OrNV infection not likely
20-1124	c744cb6b	Yigo, Trap: ac646324	13.532678	144.871081	not recorded	CRB-G	No	OrNV infection not likely