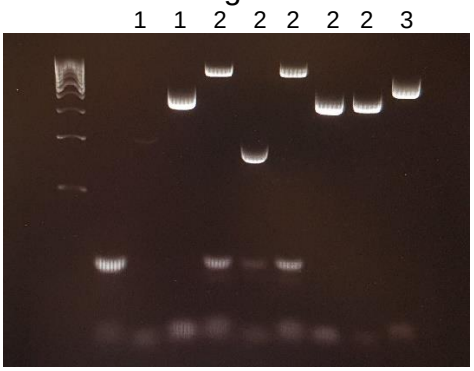
Polymerase Chain Reaction (PCR) analysis of the coconut rhinoceros beetle (CRB), *Oryctes rhinoceros* Part.1

Positive controls. So far three OrV virus isolates produced in *Heteronychus arator* (African black beetle) cell culture obtained from Sean Marshall (AgResearch, New Zealand) have been tested as positive controls in the PCR analysis of CRB population samples from Guam and Taiwan. Mixed results have been obtained with V23B, Dug42, and OrvB isolates run as positive controls when using the various published DNA oligo primer sets. In run 10/21/2019 primers **OrV2a/2b**, **OrV15a/15b**, **OrV3a/3b**, **OrV2c/2d**, and **AdV-F1/R1** detected DNA fragments as shown in the following table. Illustra PuReTaq™ Ready-to-Go PCR beads in 0.5 µl tubes were used as the PCR reaction mixture, which consists of a 25 µl reaction volume with approximately 2.5 units PuReTaq polymerase, 200 µM dNTP (dATP, dCTP, dGTP, dTTP), BSA, stabilizers and reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$). For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The resultant PCR products are subject to an electrophoresis run in a 2% Metaphor agarose gel in TBE buffer along with 100 bp DNA ladder as a size marker. Gels will be stained with either ethidium bromide or Gel Green Safe™ DNA gel stain. The red numbers indicate agreement with what fragment size is expected from the primers (Table 1).

Table 1

	Primer set	2a/2b	15a/15b	3a/3b	2c/2d	AdV-F1/R1
	Expected fragment (bp) ➡	**481**	**945**	**212**	**504**	**644**
	Virus isolates					
1	V23B	481				
2	Dug42	945	945	150	480	480
3	OrVB		500			

Fig. 1



Amplification was performed employing the following conditions: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 57^0C for 30 s, and 72^0C for 45 s; and (3) a final extension at 72^0C for 45 s. The only primers that detected the correct amplicon size was 2a/2b for V23B and 15a/15b for the Dug42 isolate. There is no obvious

explanation for the observed discrepancies at this point in the analysis. Table 2 is an amplification from 10-22-2019. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. Both virus isolates showed DNA amplicon sizes as expected from the primers used. The red numbers in the table indicate agreement with what fragment size is expected from the primers.

Table 2

	Primer set	2a/2b	15a/15b	3a/3b	2c/2d
	Expected fragment (bp) ➡	**481**	**945**	**212**	**504**
	Virus isolates				
1	Dug42	481	945	212	504
2	OrVB	481	945		

Fig. 2

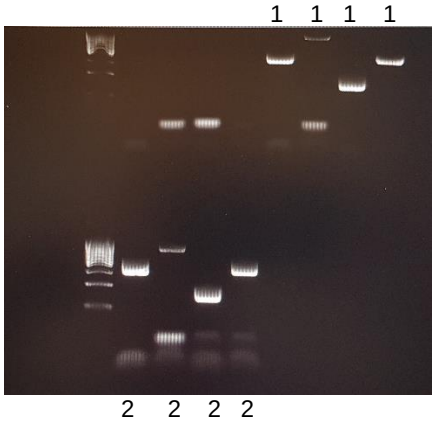


Table 3 is an amplification from 10-24-2019. Amplification was performed with primer sets **OrV-2c/2d** and **OrV-3a/3b** on only OrVB isolate employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The DNA amplicons were somewhat in ca. the same position as expected, but this run needs to be repeated for further verification.

Table 3

	Primer set	2c/2d	3a/3b
	Expected fragment (bp) ➡	**504**	**212**
	Virus isolates	-	-
1	Negative control	-	-
2	Negative control	-	-
3	OrvB	504	
4	OrVB		212

Fig. 3

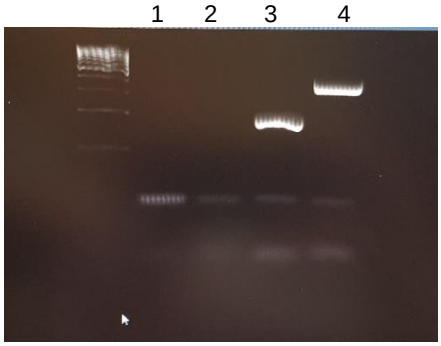


Table 4 is an amplification employing primer set **OrV-2a/2b** from 10-25-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 57^0C for 30 s, and 72^0C for 45 s; and (3) a final extension at 72^0C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. Only the positive control (V23B) showed amplification of the expected amplicon size. Some of the other samples showed unexpected amplifications.

Table 4

	Primer set	2a/2b
	Expected fragment (bp) ➡	**481**
2	V23B virus from cell culture	481
3	1848 LB V23B virus treated	-
4	1640 LB V23B virus treated ♂	< 100
5	1650 LB V23B virus treated ♂	< 100
6	1815 LB V23B virus treated ♂	-
7	1655 LB V23B virus treated ♀	-
8	1616 LB V23B virus treated ♂	< 100
9	Taichung Co. 03	< 100
10	Taichung Co. 02	-

Fig. 4a

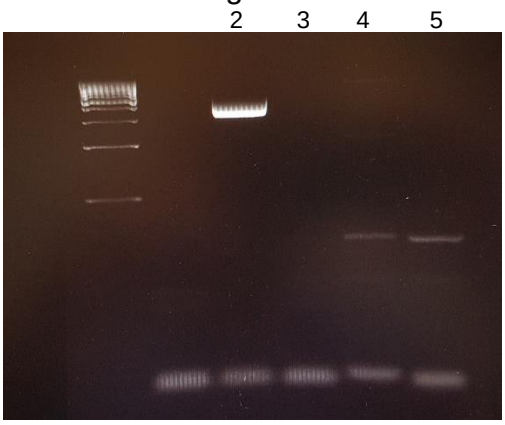


Fig. 4b

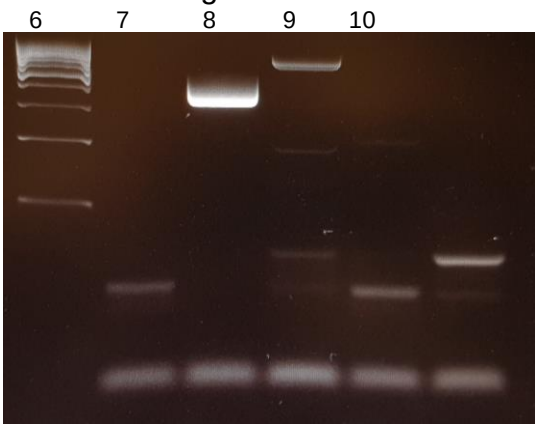


Table 5 is an amplification employing primer set **OrV-2a/2b** from 10-28-2019. Amplification was performed employing the following conditions: (1) Denaturation at

94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The only sample that showed the expected amplicon size was the positive control V23B isolate. All the other samples demonstrated multiply bands less than 150 bp except Kaohsiung City 02 which had a faint band around the 481 bp size marker. There is no obvious explanation for the observed discrepancies at this point in the analysis.

Table 5

	Primer set	2a/2b
	Expected fragment (bp) ➡	**481**
2	V23B virus from cell culture	481
3	Taichung Co. 01	-
4	Taichung Co. 02	< 100
5	1798 Taichung Co.	< 100
6	Taichung Co. 03	-
7	Taichung Co. 09	-
8	Taichung Co. 10	< 100
9	Kaohsiung City 02	< 100, 481 (f)
10	1790 TLB Taichung Co. ♂	-

(f) – faint band; TLB – treated laboratory beetle from population on Guam

Fig. 5

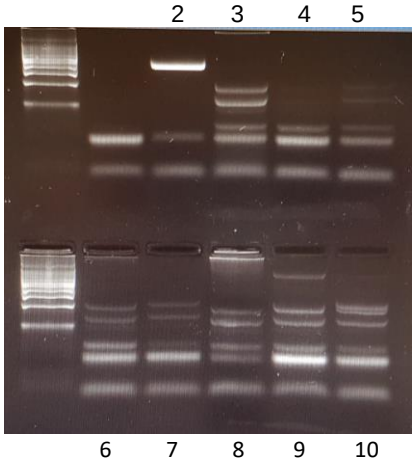


Table 6 is an amplification employing primer set **OrV-2a/2b** from 10-29-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The only sample that showed the expected amplicon size was the positive control V23B isolate.

Fig. 6

	Primer set	2a/2b
	Expected fragment (bp) ➡	**481**
2	V23B virus from cell culture	481
3	1716a TLB Kaohsiung City mg prep	-
4	1743a TLB Kaohsiung City ♂ mg prep	< 100
5	1683b TLB Kaohsiung City ♂ mg prep	< 100
6	1808b TLB Kaohsiung City ♂ mg prep	-
7	Kaohsiung City 02	< 100
8	Kaohsiung City 03	< 100
9	1800 TLB Kaohsiung City ♂ mg prep	-
10	negative control	-

Fig. 6

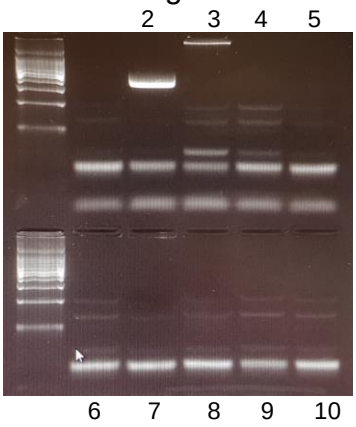


Table 7 is an amplification employing primer set **OrV-3a/3b** from 10-30-2019A. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The only sample that showed the expected amplicon size was the positive control Dug42 isolate.

Table 7

	Primer set	3a/3b
	Expected fragment (bp) ➡	**212**
2	Dug42 virus from cell culture	212
3	1716a TLB Kaohsiung City mg prep	-
4	1743a TLB Kaohsiung City ♂	-
5	1683b TLB Kaohsiung City ♂ mg prep	-
6	1808b TLB Kaohsiung City ♂ mg prep	-
7	Kaohsiung City 02	-
8	Kaohsiung City 03	-
9	1800 TLB Kaohsiung City ♂ mg prep	-
10	Kaohsiung City 05	-

TLB – treated laboratory beetle samples from population on Guam
mg prep – midgut preparation from Taiwan beetle

Fig. 7

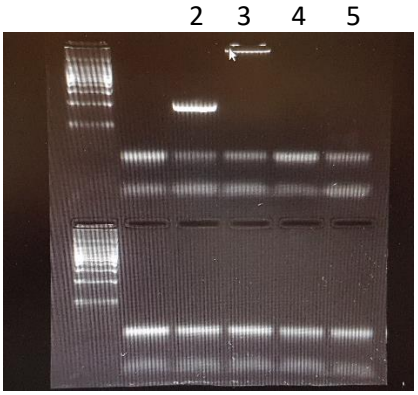


Table 8 is an amplification employing primer set **OrV-2a/2b** from 10-30-2019B. Amplification was performed employing the following conditions: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 57^0C for 30 s, and 72^0C for 45 s; and (3) a final extension at 72^0C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The positive control V23B virus isolate showed a strong amplicon band while 1659, 1639, and 1597 samples showed the expected amplicon but the bands were faint.

Table 8

	Primer set	2a/2b
	Expected fragment (bp) ➡	**481**
2	V23B virus from cell culture	481
3	1651 V23B perOS ♀	481 (f)
4	1639 V23B perOS ♂	481 (f)
5	1605 V23B perOS ♂	-
6	1609 V23B perOS ♂	-
7	1597 V23B perOS ♀	481 (f)
8	1633 V23B perOS ♂	-
9	1659 V23B perOS ♂	-
10	1617 V23B control per OS ♀	

(f) – faint band

Fig. 8

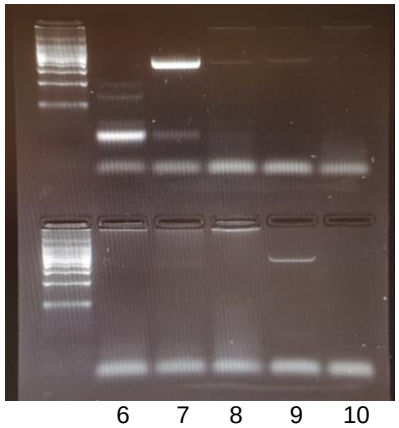


Table 9 is an amplification employing primer set **OrV-2a/2b** from 11-04-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The positive control V23B virus isolate showed a strong amplicon band. Only samples 1609 V23B perOS ♂ and 1659 V23B perOS ♂ produced an amplicon indicating the presence of virus in the beetles that were orally treated with V23B virus preparation.

Table 9

	Primer set	2a/2b
	Expected fragment (bp) ➡	**481**
2	V23B virus from cell culture	481
3	1651 TLB V23B perOS ♀	-
4	1639 TLB V23B perOS ♂	-
5	1605 TLB V23B perOS ♂	-
6	1609 TLB V23B perOS ♂	481
7	1597 TLB V23B perOS ♀	-
8	1633 TLB V23B perOS ♂	-
9	1659 TLB V23B perOS ♂	481 (f)
10	1617 TLB V23B control per OS ♀	-

(f) – faint band; TLB – treated laboratory beetle samples from population on Guam

Fig. 9

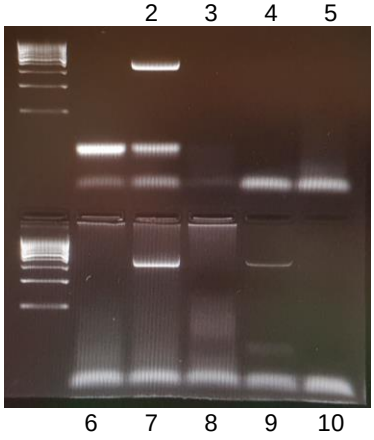


Table 10 is an amplification employing primer set **OrV-15a/15b** from 11-05-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The positive control V23B virus isolate showed a strong amplicon band. Only sample 1659 V23B perOS ♂ produced a faint amplicon indicating the presence of virus in the beetles that was orally treated with V23B virus preparation.

Table 10

	Primer set	15a/15b
	Expected fragment (bp) ➡	**945**
2	V23B virus from cell culture	945
3	1639 TLB V23B perOS ♂	-
4	1617 TLB V23B control per OS ♀	
5	1618 TLB V23B perOS ♂	-
6	1804 TLB V23B perOS ♀	-
7	1846 TLB V23B perOS ♂	-
8	1854 TLB V23B perOS ♀	-
9	1609 TLB V23B perOS ♂	-
10	1659 TLB V23B perOS ♂	945 (f)

(f) – faint band; TLB – treated laboratory beetle samples from population on Guam

Fig. 10

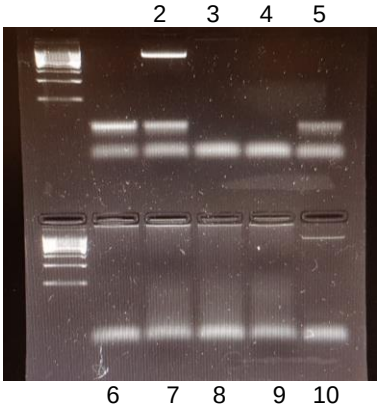


Table 11 is an amplification employing primer set **OrV-2a/2b** from 11-05-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The positive control V23B virus isolate showed a strong amplicon band. All other samples were negative for DNA virus detection.

Table 11

	Primer set	2a/2b
	Expected fragment (bp) ➡	**481**
2	V23B virus from cell culture	481
3	1716a ♂ TLB w/ Kaohsiung City mg prep.	-
4	1683b ♂ TLB Kaohsiung City mg prep.	
5	Kaohsiung City 05	-
6	1800 ♂ TLB Kaohsiung City mg prep.	-
7	1808b ♂ TLB Kaohsiung City mg prep.	-
8	Kaohsiung City (°°)	-
9	Kaohsiung City (++)	-
10	Kaohsiung City (°°°°)	-

mg prep – midgut preparation from Taiwan beetles

Fig. 11

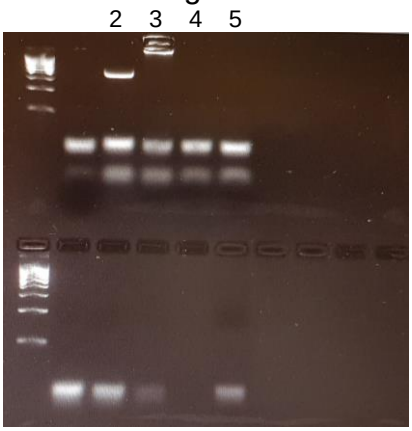


Table 12 is an amplification employing primer set **OrV-15a/15b** from 11-06-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 57^0C for 30 s, and 72^0C for 45 s; and (3) a final extension at 72^0C for 45 s. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. The positive control V23B virus isolate showed a strong amplicon band. All other samples were negative for DNA virus detection.

Table 12

	Primer set	2a/2b
	Expected fragment (bp) ➡	**945**
2	V23B virus from cell culture	945
3	1716a ♂ TLB w/ Kaohsiung City mg prep.	-
4	1683b ♂ TLB Kaohsiung City mg prep.	
5	Kaohsiung City 05	-
6	1800 ♂ TLB Kaohsiung City mg prep.	-
7	1808b ♂ TLB Kaohsiung City mg prep.	-
8	Kaohsiung City (°°)	-
9	Kaohsiung City (++)	-
10	Kaohsiung City (°°°°)	-

mg prep – midgut preparation from Taiwan beetle

Fig. 12

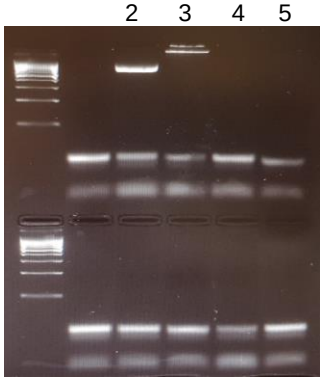


Table 13 is an amplification employing primer sets **AdV-F1/R1**, **Adv-F2/R2**, and **Adv-F3/R3** from 11-06-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s with 5 µl DNA template, 1 µl primers, 19 µl nuclease-free water. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. These primers amplify a similar amplicon size so it is difficult to separate the difference among them on a gel. V23B virus isolate was used as the positive control.

Table 13

	Primer set	AdV-F1/R1	AdV-F2/R2	AdV-F3/R3
	Expected fragment (bp) ➡	**644**	**605**	**644**
2	V23B virus from cell culture	644		
3	Dug42 virus from cell culture	644		
4	V23B virus from cell culture		605	
5	Dug42 virus from cell culture		605	
6	OrVB virus from cell culture		605	
7	V23B virus from cell culture			644
8	Dug42 virus from cell culture			644
9	OrVB virus from cell culture			644

Fig. 13

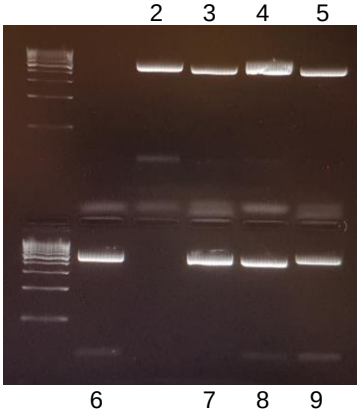


Table 14 is an amplification employing primer sets **AdV-F1/R1**, **Adv-F2/R2**, and **Adv-F3/R3** from 11-08-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s with 5 µl DNA template, 1 µl primers, 19 µl nuclease-free water. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. V23B virus isolate was used as the positive control.

Table 14

	Primer set	Adv/F1/R1	Adv-F2/R2	Adv-F3/R3
2	Expected fragment (bp) ➡	**644**	**605**	**644**
3	V23B virus from cell culture	644		
5	Dug42 virus from cell culture	644		
6	OrVB virus from cell culture	-		
7	V23B virus from cell culture		605	
8	Dug42 virus from cell culture		605	
9	OrVB virus from cell culture		605	
10	V23B virus from cell culture			644
11	Dug42 virus from cell culture			644
12	OrVB virus from cell culture			644

Fig. 14

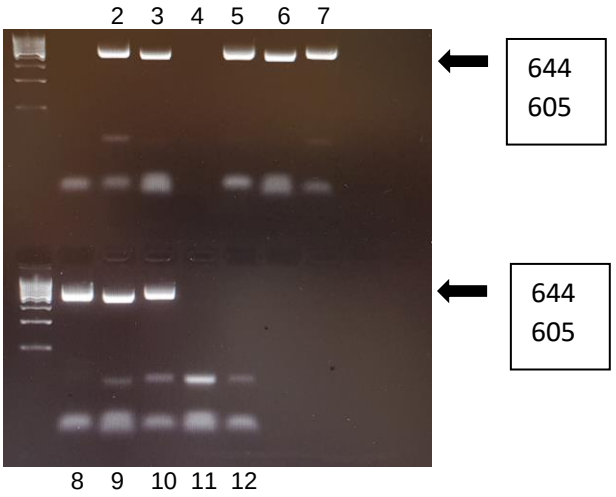


Table 15 is an amplification employing primer set **AdV-F1/R1** from 11-18-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s with 5 µl DNA template, 1 µl primers, 19 µl nuclease-free water. For the negative control 1 µl primer and 24 µl nuclease-free water and positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water was used for amplification. This is an interesting amplification as the primer used indicated that there was virus present in all the samples except 1800 TLB Kaohsiung V23B mg prep. However, the control sample also showed an indication of virus presence. This might indicate that either the beetles collected on Guam have a covert viral presence in their system or a cross-contamination occurred in the preparation of the control sample prior to the PCR amplification. V23B virus isolate was used as the positive control.

Table 15

	Primer set	Adv/F1/R1
	Expected fragment (bp) ➡	**644**
2	V23B virus from cell culture	644
3	1651 V23B virus ♀	644
4	1639 V23B virus ♀	644
5	1639 V23B virus ♂	644
6	1597 V23B virus ♀	644
7	? 1633 V23B virus ♂	644
8	1659 V23B virus ♂	644
9	1800 TLB Kaohsiung mg prep ♂	-
10	1808b TLB Kaohsiung mg prep ♂	644
11	1617 TLB V23B control ♀	644
12	1609 TLB V23B virus ♂	644

mg prep – midgut preparation from Taiwan beetles TLB – treated laboratory beetle samples from population on Guam

Fig. 15

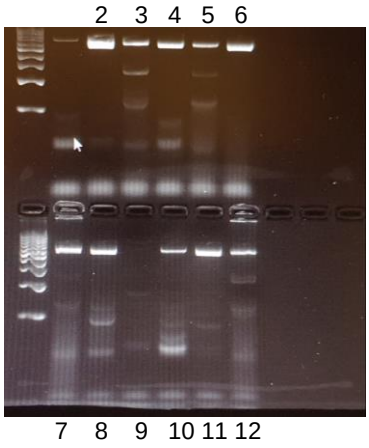


Table 16 is an amplification employing primer set **AdV-F1/R1** from 11-19-2019A. This is a repeat amplification of samples from 11-18-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s with 5 µl DNA template, 1 µl primers, 19 µl nuclease-free water. For the negative control 1 µl primer and 24 µl nuclease-free water and in the positive control 1 µl primer, 1-3 ul DNA virus template and 21-23 µl nuclease-free water were used for amplification. V23B virus isolate was used as the positive control.

Table 16

	Primer set	Adv/F1/R1
	Expected fragment (bp) ➡	**644**
2	V23B virus from cell culture	644
3	1651 V23B virus ♀	644
4	1639 V23B virus ♀	644 (f)
5	1605 V23B virus ♂	644 (f)
6	1597 V23B virus ♀	644
7	1633 V23B virus ♂	644

8	1659 V23B virus ♂	644
9	1800 TLB Kaohsiung mg prep ♂	-
10	1808b TLB Kaohsiung mg prep ♂	-
11	1617 TLB V23B control ♀	-
12	1609 TLB V23B virus ♂	-

This is the second amplification for the same sample set as shown in Table 15 employing primers **AdV-F1/R1A**. Notice this time there was no amplification of the expected amplicon (644 bp) in samples 9, 10, and 11. For sample 3, the expected amplicon (644 bp) was detected; however, the band was faint. For a second time, the expected amplicon was not detected in sample 1800. For samples 1639, 1597, and 1659, a number of smaller unknown secondary bands were amplified at ca. the 280, 200, and 150 bp marker.

Fig. 16

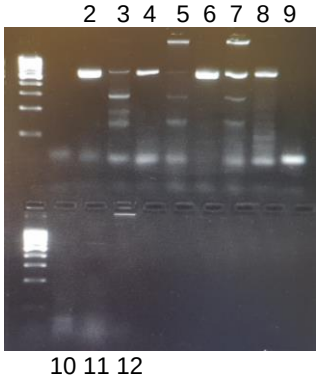
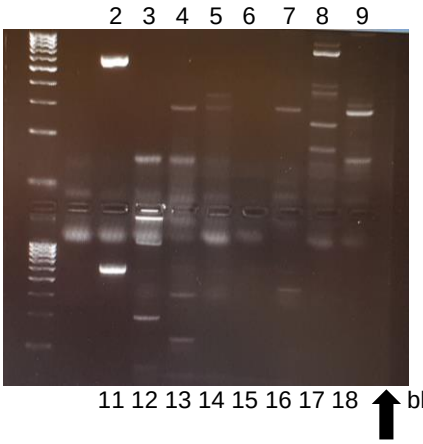


Table 17 is an amplification employing primer set **AdV-F1/R1B** from 11-19-2019B. This is a repeat amplification of samples from 11-18-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 45 s with 5 µl DNA template, 1 µl primers, 19 µl nuclease-free water. V23B virus isolate was used as the positive control.

Table 17

	Primer set	Adv/F1/R1
	Expected fragment (bp) ➡	**644**
2	V23B virus from cell culture	644
3	Taichung Co. 01	150
4	Taichung Co. 02	150, 300
5	Taichung Co. 03	300, 350
6	Taichung Co. 09	-
7	Taichung Co. 10	280
8	1790 TLB Taichung City mg prep ♂	150, 200, 350, 400, 644
9	6-3a TLB Kaohsiung City mg prep ♂	150, 280
10		
11	1743b TLB Kaohsiung City mg prep ♂	-
12	1668 TLB Kaohsiung City mg prep ♂	644
13	1798 TLB Kaohsiung City mg prep ♀	200 (f)
14	Kaohsiung City 05	-
15	Kaohsiung City 03	-
16	Kaohsiung City (°°°°)	300 (f)
17	Kaohsiung City 02	300 (f)
18	Kaohsiung City (°°°)	-

Fig.17



For samples in Table 17 Taichung Co. 01, Taichung Co. 02, Taichung Co. 03, 1790 TLB Taichung City mg prep ♂, Taichung Co. 10 and, 6-3a TLB Kaohsiung City mg prep ♂ a number of smaller unknown secondary bands were amplified at ca. the 350, 280, 200, and 150 bp markers. For samples No.10, 13, 14, and 17 a number of smaller unknown secondary bands were also amplified at ca. the 100, 150, and 300 bp markers. Samples No. 2, 8, and 12 amplified the expected amplicon size of ca. 644 bp.

Table 18 is an amplification employing primer set **OrV-15a/15b** from 11-21-2019. Amplification was performed employing the following conditions, which are different than the previous amplifications. (1) Denaturation at 94^0C for 1 min; (2) 35 cycles of 94^0C for 30 s, 50^0C for 45 s, and 72^0C for 1 min; and (3) a final extension at 72^0C for 5 min with 5 µl DNA template, 1 µl primers, 19 µl nuclease-free water. V23B virus isolate as the positive control showed a strong amplicon band at the expected bp marker.

Table 18

	Primer set	OrV-15a/15b
	Expected fragment (bp) ➡	**945**
2	V23B virus from cell culture	-
3	Taichung Co. 01	-
4	Taichung Co. 02	-
5	Taichung Co. 03	-
6	Taichung Co. 09	-
7	Taichung Co. 10	> 945
8	1790 TLB Taichung City mg prep ♂	-
9	6-3a TLB Kaohsiung City mg prep ♂	-
10		-
11	1743b TLB Kaohsiung City mg prep ♂	-
12	1668 TLB Kaohsiung City mg prep ♂	-
13	1798 TLB Kaohsiung City mg prep ♀	> 945
14	Kaohsiung City 05	-
15	Kaohsiung City 03	-
16	Kaohsiung City (°°°°)	-
17	Kaohsiung City 02	-
18	Kaohsiung City (°°°)	-

Fig. 18

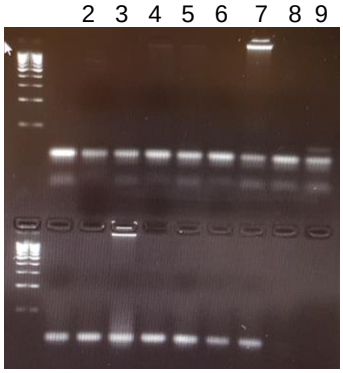


Table 19 is an amplification employing primer set **OrV-15a/15b** from 11-25-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 50^{0}C for 45 s, and 72^{0}C for 1 min; and (3) a final extension at 72^{0}C for 5 min with 5 µl DNA template, 1 µl primers, 19 µl nuclease-free water. Dug42 virus isolate as the positive control showed a strong amplicon band at the expected bp marker.

Table 19

	Primer set	OrV-15a/15b
	Expected fragment (bp) ➡	**945**
2	Dug42 virus from cell culture	945
3	1605 LB	> 945
4	1824 LB	> 945
5	1922 LB	-
6	1597 LB	945
7	1633 LB	945 (f)
8	1659 LB	945
9	1800 TLB Kaohsiung City mg prep ♂	-
10	1808b TLB Kaohsiung City mg prep ♂	-
11	1617 LB	945
12	1646 LB	945
13	1800 LB	> 945
14	1685 TLB Taichung Co. mg prep ♀	> 945
15	1806 LB	> 945
16	1651 LB	> 945
17	1639 LB	> 945 (f)
18	1609 LB	> 945

LB – laboratory beetle collected from Guam population; (f) – faint band

Fig. 19

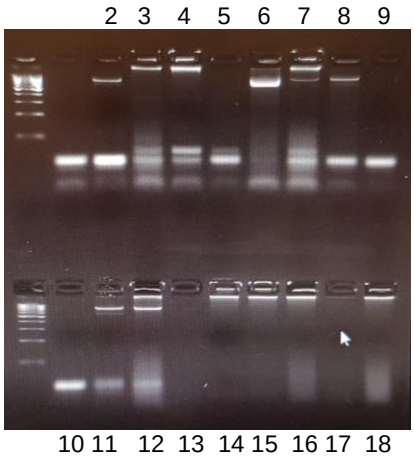
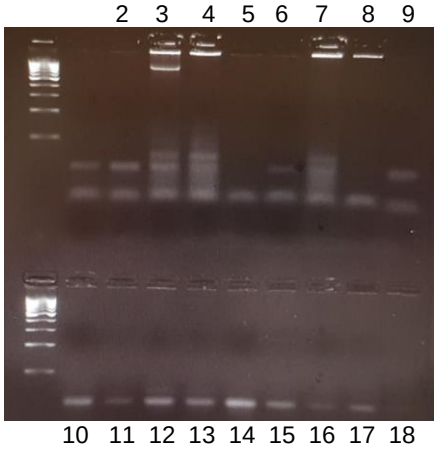


Table 20 is an amplification employing primer set **OrV-15a/15b** from 11-26-2019. Amplification was performed employing the following conditions: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 50^{0}C for 45 s, and 72^{0}C for 1 min; and (3) a final extension at 72^{0}C for 5 min with 5 µl DNA template, 1 µl primers, 19 µl nuclease-free water. Dug42 virus isolate as the positive control showed no amplicon at the expected bp marker. The remaining samples either showed no amplification or had amplicons larger than the expected 945 bp marker.

Table 20

	Primer set	OrV-15a/15b
	Expected fragment (bp) ➡	**945**
2	Dug42 virus from cell culture	-
3	1597 LB	> 945, 945
4	1633 LB	> 945
5	1659 LB	-
6	1617 LB	-
7	1646 LB	> 945
8	1716a TLB Kaohsiung City mg prep ♂	> 945
9	Kaohsiung City (°°)	-
10	Kaohsiung City (++)	-
11	1683b TLB Kaohsiung City mg prep ♂	-
12	1743a TLB Kaohsiung City mg prep ♂	-
13	1790 Taichung Co. ♂	-
14	Kaohsiung City (°°°)	-
15	Kaohsiung City 02	-
16	Kaohsiung City (°°°°)	-
17	Kaohsiung City 03	-
18	Kaohsiung City 05	-

Fig. 20



DNA oligo primers.

(1) [First Report of Oryctes rhinoceros nudivirus (Coleoptera: Scarabaeidae) Causing Severe Disease in *Allomyrina dichotoma* in Korea. Seokhyun Lee, Kwan-Ho Park, Sung-Hee Nam, Kyu-Won Kwak, and Ji-Young Choi. 2015. **J Insect Sci.** 15(26): DOI: 10.1093/jisesa/iev002]

OrNV genome (GenBank accession no. NC_011588).
Primer AdV-F1 is 5'-TCCGGAAATTACACGA GCCAC-3'
Primer AdV-R1 is 5'-ATGCCGTACGAGAGTATAGGTCG-3'
Amplification using primer pair AdV-F1 and -R1 yields **644 bp** fragment of lef-8 gene (OrNV_gp064).

Primer AdV-F2 is 5'-TCGAATCCGTTTCCGATACTTACAG-3'
Primer AdV-R2 is 5'-TGAGTAGCGCTATAGACTGCTC-3'
Amplification between primer AdV-F2 and -R2 produces the **605 bp** fragment of GrBNV_gp76-like protein (OrNV_gp025).

Primer AdV-F3 is 5'-GGGTGTGACGAGAAAACA ACGC-3'
Primer AdV-R3 is 5'-GCAGGCGTGTAATAAATGGCGG-3'
Amplification between AdV-F3 and –R3 yields **644 bp** fragment of ribonucleotide reductase gene (OrNV_gp051).

(2) [Primers for the detection of *Oryctes* virus from Scarabaeidae (Coleoptera). 1999. Nicola A. K. Richards, Travis R. Glare, Ioane Aloali, and Trevor Jackson. **Mol. Ecol. Primer Notes**. 8: 1551-1561.]

Primer pair	Sequence (5' - 3')	Product size (bp)
OrV15a	ATT ACG TCG TAG AGG CAA TC	945
OrV15b	CAT GAT CGA ATT CGT CTA TGG	
OrV3a	GAA CGC AAG TTT GCA GTA TG	212
OrV3b	ATA CTA TCG TCT CCG ATG TC	
OrV2a	AGA GTC TAC GAA ATT CGA GC	481
OrV2b	CAT TTA AGC GTT TAC GTT CG	
OrV2d	ACT GCT GTT TAC GAT CGA AG	504
OrV2c	GTA CAG TTG AGC GCT AGT AC	
OrV11a	GCA GTC CCG TAG AGG ATT AC	409
OrV11b	AAG CTC CTA CCG CGA CCA CA	