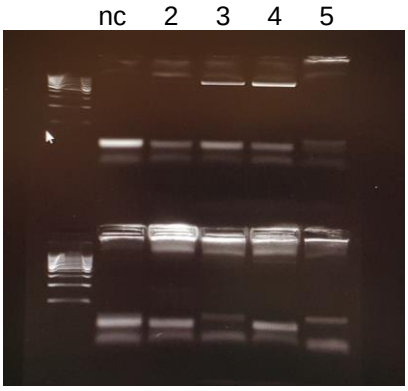
Polymerase Chain Reaction (PCR) detection and analysis of virus isolates in coconut rhinoceros beetle (CRB), *Oryctes rhinoceros* Part. 3

Amplification results of 2-26-2020 in Table 32 was performed employing the following conditions and **15a/15b** primers: (1) Denaturation at 94^0C for 3 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. 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. DNA samples (3-5 µl) were run on a 1.5% Metaphor gel (TBE buffer, pH 8.3) with a Beijing Liuyi DYY-C6 power supply at a constant 120 v, stained with ethidium bromide and photographed with a BioRad GelDoc XR+ imager. The virus isolates were expected to generated a DNA amplicon sizes of 945 bp.

Table 32

	DNA sample	15a/15b
	Expected fragment (bp) ➡	**945**
2	V23B virus from cell culture	
3	Dug42 virus from cell culture	
4	S2A virus from cell culture	
5	Taichung Co. 10	
6	C-1 1/6/2020 ♂	
7	C-36 F/M 1/10/2020 ♀	
8	C-41 IM/UF 1/27/2020 ♀	
9	1659 LB	
10	C-45 M/F 2/6/2020 ♀	

Fig. 32



Some of the samples showed amplification including two of the positive controls; however, the size of the DNA amplicons was difficult to determine and appeared to be somewhat smaller than the expected fragments as predicted from the 15a/15b primers.

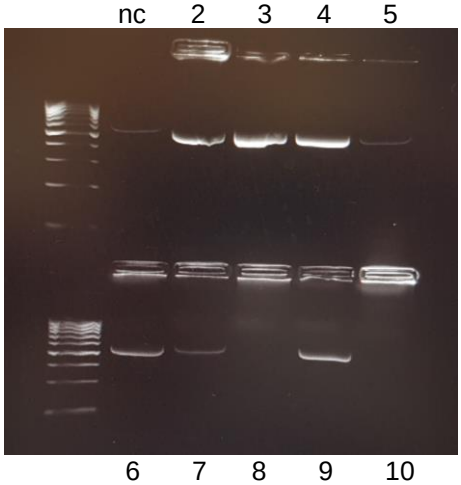
PCR amplification results of 2-27-2020 in Table 33 was performed employing the following running conditions and **AdV-F1/R1** primers: (1) Denaturation at 95^{0}C for 3 min; (2) 35 cycles of 94^{0}C for 30 s, 57^{0}C for 30 s, and 72^{0}C for 45 s; and (3) a final extension at 72^{0}C for 10 min. Illustra 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. DNA samples (5 µl) were run on a **1.5%** Metaphor gel (TBE buffer, pH 8.3) with a Beijing Liuyi DYY-6C power supply at a constant 120 v. The gel was stained with Gel Green (Caroline Biological Supply) and photographed using a BioRad GelDoc XR+ imager. The virus isolates were expected to generated DNA amplicon sizes of 644 bp.

Table 33

(f) - faint

	DNA sample	Adv-F1/R1
	Expected fragment (bp) ➡	**644**
2	V23B virus from cell culture	500
3	PNG virus from cell culture	500
4	S2A virus from cell culture	500
5	Taichung Co. 10	500 (f)
6	C-1 1/6/2020 ♂	500
7	C-36 F/M 1/10/2020 ♀	500 (f)
8	C-41 IM/UF 1/27/2020 ♀	-
9	1659 LB	500
10	C-45 M/F 2/6/2020 ♀	-

Fig. 33



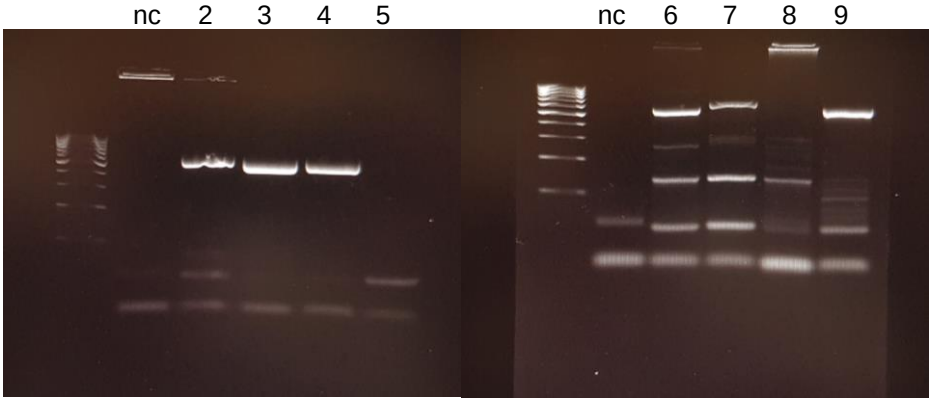
Many of the samples showed amplification; however, the fragments were smaller than expected appearing around 400 and 500 bp rather than 644 bp. The negative control was contaminated so this PCR run will have to be repeated.

PCR amplification results of 2-28-2020 in Table 34 was performed employing the following running conditions and **AdV-F1/R1** primers: (1) Denaturation at 95^{0}C for 3 min; (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 10 min. 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. DNA samples (5 µl) were run on a **1.5%** Metaphor gel (TBE buffer, pH 8.3) with a Beijing Liuyi DYY-6C power supply at a constant 120 v. The gel was stained with Gel Green (Caroline Biological Supply) and photographed using a BioRad GelDoc XR+ imager. The virus isolates were expected to generated DNA amplicon sizes of 644 bp.

Table 34

	Primer set	AdV-F1/R1
	Expected fragment (bp) ➡	**644**
2	V23B virus from cell culture	644
3	Dug42 virus from cell culture	644
4	S2A virus from cell culture	644
5	Taichung Co. 10	-
6	C-1 1/6/2020 ♂	644
7	C-36 F/M 1/10/2020 ♀	644
8	C-41 IM/UF 1/27/2020 ♀	-
9	1659 LB	644

Fig. 34



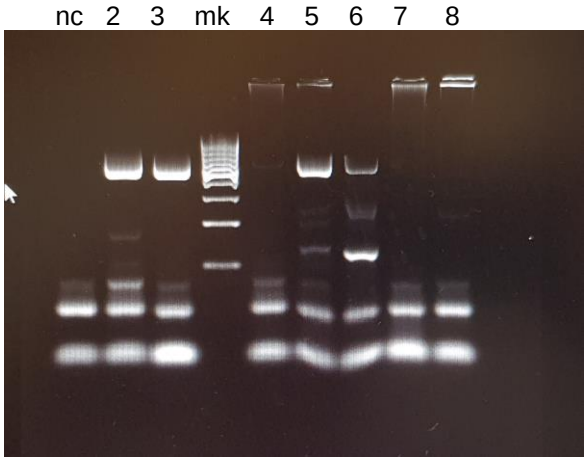
This I suppose was a good amplification (Fig. 340. All three positive controls showed as well as there was no contamination in the negative control. The AdV F1/R1 primers amplified close to the expected bp fragment for C-1 1/6/2020 ♂, C-36 F/M 1/10/2020 ♀, and 1659 LB samples.

PCR amplification results of 3-5-2020 in Table 35 was performed employing the following running conditions and **AdV-F1/R1** primers: (1) Denaturation at 95^{0}C for 3 min; (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 10 min. 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. DNA samples (5 µl) were run on a **1.5%** Metaphor gel (TBE buffer, pH 8.3) with a Beijing Liuyi DYY-6C power supply at a constant 120 v. The gel was stained with Gel Green (Caroline Biological Supply) and photographed using a BioRad GelDoc XR+ imager. The virus isolates were expected to generated DNA amplicon sizes of 644 bp.

Table 35

	Primer set	AdV-F1/R1
	Expected fragment (bp) ➡	**644**
1	negative control (nc)	-
2	V23B virus from cell culture	600
3	S2A virus from cell culture	600
	DNA bp marker (mk)	-
4	Taichung Co. 10	600 (f)
5	C-1 1/6/2020 ♂	600
6	C-36 F/M 1/10/2020 ♀	600
7	C-41 IM/UF 1/27/2020 ♀	-
8	C-45 M/F 2/6/2020 ♀	-

Fig. 35



Both positive controls were amplified while the negative control was clean. Sample No. 4 (Taichung Co. 10), sample No. 5 (C-1(1/6/2020) ♂), and sample No. 6 (C-36 F/M (1/10/2020) ♀) showed an approximate fragment size of 600 bp similar to what was

expected when employing the AdV-F1/R1 primers. Furthermore, Sanger DNA sequencing will be required to confirm if indeed virus is present in the beetles.