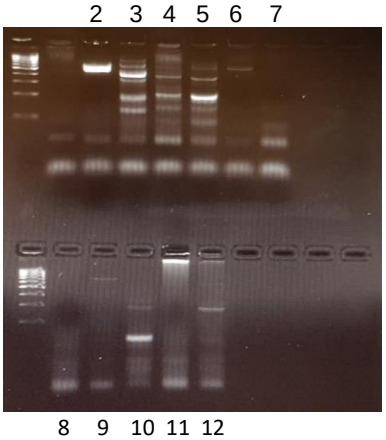
Polymerase Chain Reaction (PCR) detection and analysis of virus isolates in coconut rhinoceros beetle (CRB), *Oryctes rhinoceros* Part. 2

Amplification results of 12-05-2019 in Table 21 was performed employing the following conditions and AdV-F1/R1 primers: (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. 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. DNA samples (5 µl) were run on a 2% 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 V23B virus isolate generated as expected a DNA amplicon sizes of 644 bp.

Table 21

	Primer set	AdV-F1/R1
	Expected fragment (bp) ➡	**644**
2	V23B virus from cell culture	644 (f)
3	Kaohsiung City 02	-
4	Kaohsiung City 03	-
5	Kaohsiung City 05	644
6	Kaohsiung City (°°)	-
7	Kaohsiung City (°°°°)	-
8	Kaohsiung City (°°°)	-
9	Kaohsiung City (++)	
10	Taichung Co. 01	-
11	Taichung Co. 10	-
12	Taichung Co. 03	

Fig. 21



PCR amplification results of 12-10-2019 in Table 22 was performed employing the following conditions and **AdV-F1/R1** primers: (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. Table 22 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. DNA samples (5 µl) were run on a 2% Metaphor gel (TBE buffer, pH 8.3) with a Beijing Liuyi DYY-6C power supply at a constant 120 v, stained with ethidium bromide and photographed with a BioRad GelDoc XR+ imager. The V23B virus isolate generated a DNA amplicon sizes (644 bp) as expected thought it was a faint band. The red numbers in the table indicate agreement with what fragment size is expected from the primers used. Of all the samples used in the bioassay of 7-7-2019 that actually died during the test, only sample 1597 LB ♀ V23B virus treated beetle generated a strong band at the expected size range, indicating possible virus-caused mortality.

Table 22

	Primer set	AdV-F1/R1
	Expected fragment (bp) ➡	**644**
2	V23B virus from cell culture	644 (f)
3	1651 TLB V23B virus treated ♀	-
4	1640 TLB V23B virus treated ♂	-
5	1597 TLB V23B virus treated ♀	644
6	1633 TLB V23B virus treated ♂	-
7	1609 TLB V23B virus treated ♂	-
8	1808b TLB Kaohsiung City mg prep ♂	-
9	1685 TLB Kaohsiung City mg prep ♀	-
10	1800 TLB Kaohsiung City mg prep ♂	-

TLB – laboratory beetle sampled from wild Guam population
(f) – faint band; mg prep – midgut preparation

Fig. 21a

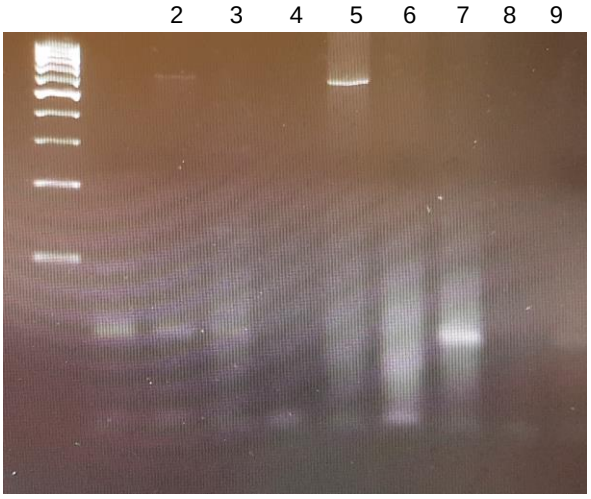
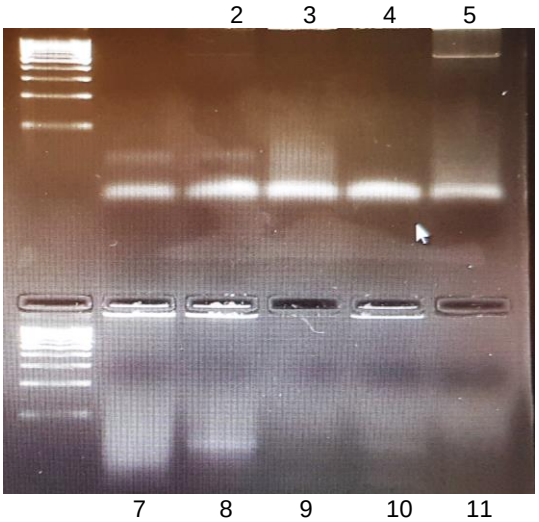


Fig. 21b



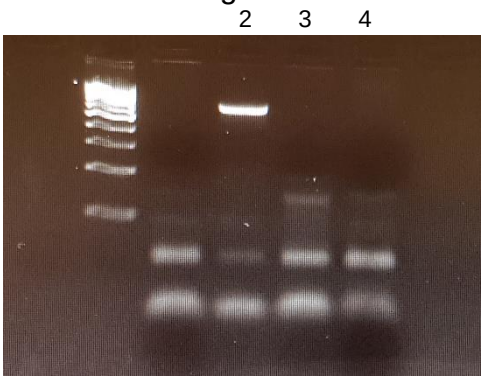
In a previous amplification of 11-18-2019 (Fig. 15) using the AdV-F1/R1 primers, all of the samples depicted in the above Table 22 that were used in bioassay of 7-7-2019

2019 that actually died during the test showed a strong amplicon band at the expected size of 644 bp, indicating also possible virus-caused mortality.

Table 23

	Primer set	AdV-F1/R1
	Expected fragment (bp) ➡	**644**
2	V23B virus from cell culture	644
3	1648 TLB V23B virus per OS ♂	-
4	1616 TLB V23B virus per OS ♂	-

Fig. 23



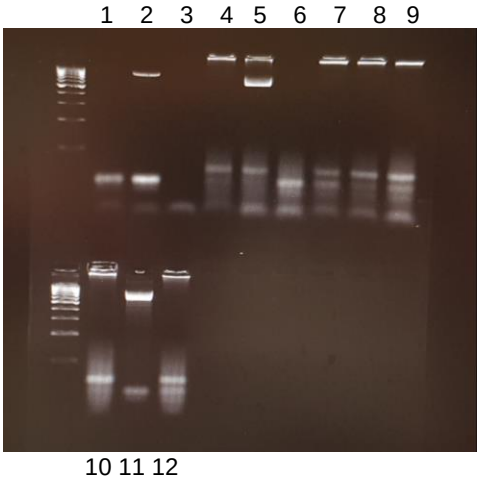
PCR amplification results of 12-10-2019 in Table 24 was performed employing the following conditions and **15a/15b** primers: (1) Denaturation at 94^{0}C for 30 s; (2) 35 cycles of 94^{0}C for 30 s, 50^{0}C for 45 s, and 72^{0}C for 1min; and (3) a final extension at 72^{0}C for 5 min. For the negative 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. Samples tested were from an autodissemination study to determine the potential transmission of virus by laboratory infected adult beetles to non-infected adults.

Table 24

Primer set	Sample No.	Sample description	expected fragment (bp)
15a/15b	1	negative control	-
"	2	V23B virus cell culture	➡ **945**
"	3	C-23 F/M 1/10/2020 ♀	ca. 945
"	4	C-29 F/M 1/10//2020 ♀	-
"	5	C-36 F/M 1/10/2020 ♀	ca. 945
"	6	T-1 IM/UF male 1/2/2020 ♀	-
"	7	T-7 IF/UM 12/29/19 ♂	-
"	8	T-10 IM/UF 1/8/2020 ♂	-
"	9	C-7 1/2/2020 ♀	-
"	10	C-7 1/2/2020 ♂	-
"	11	T-13 IF/UM 1/8/2020 ♀	ca. 945
"	12	T-9 IM/UF 1/8/2020 ♀	-

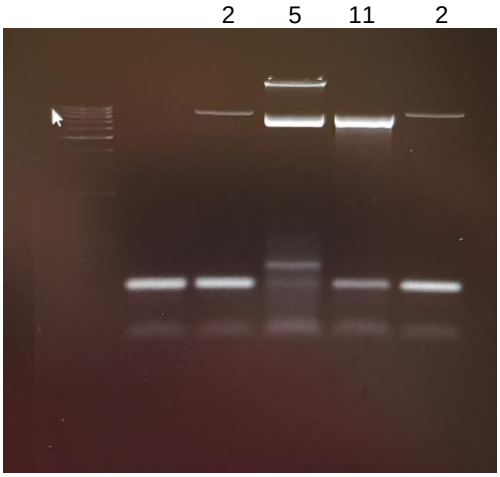
DNA samples were run on a 2% Metaphor gel (TBE buffer, pH 8.3) with a Beijing Liuyi DYY-6C power supply at a constant 120 v, stained with ethidium bromide and photographed with a BioRad GelDoc XR+ imager. V23B virus isolate generated a DNA amplicon size (945 bp) as expected thought it was a faint band.

Fig. 24a



The results are somewhat surprising (Fig.24a). One of the controls and treated sample showed an amplification around 945 bp; however, the fragment sizes were actually somewhat smaller than expected. This lends itself to the question as to whether or not the virus was detected employing the 15a/15b primers or something else entirely. The positive control showed the expected size band.

Fig. 24b



I repeated the gel electrophoresis run with the previous amplified samples 2, 5, 11 from Table 24; Fig.24a on a 2% Metaphor gel employing wider sample wells. This gave me a better delineation of the distance bands migrated through the gel.

PCR amplification results of 1-22-2020 in Table 25 was performed employing the following running conditions and **15a/15b** primers: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 50^0C for 45 s, and 72^0C for 1min; and (3) a final extension at 72^0C for 5 min. Samples tested were from an autodissemination study to determine the potential transmission of virus by laboratory infected adult beetles to non-infected adults. 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 2% Metaphor gel (TBE buffer, pH 8.3) with a Beijing Liuyi DYY-6C power supply at a constant 120 v. V23B virus isolate generated the expected DNA amplicon size (945 bp). The gel was stained with Gel Green (Caroline Biological Supply) and photographed using a BioRad GelDoc XR+ imager.

Table 25

Primer set	Sample No.	Sample description	expected fragment (bp)
15a/15b	1	negative control	
"	2	V23B virus cell culture ➡	**945**
"	3	C-3 1/19/2020 ♂	-
	4	**C-1 1/16//2020** ♂	**945**
"	5	C-32 M/F 1/19/2020 ♂	-
"	6	C-10 1/19/2020 ♀	-
"	7	C-6 1/16/2020 ♀	300/600
"	8	T-5 IM/UF 1/19/2020 ♀	-
"	9	T-15 IM/UF 1/19/2020 ♀	-
"	10	T-5 IF/UM 1/19/2020 ♂	-
"	11	**T-12 IM/UF 1/19/2020** ♂	**945**
"	12	T-3 IM/UF 1/19/2020 ♂	-
"	13	T-6 IF/UM 1/16/2020 ♀	-
"	14	**T-5 IM/UF 1/19/2020** ♂	**945**
"	15	T-10 IF/UM 1/19/2020 ♀	-

Fig. 25a

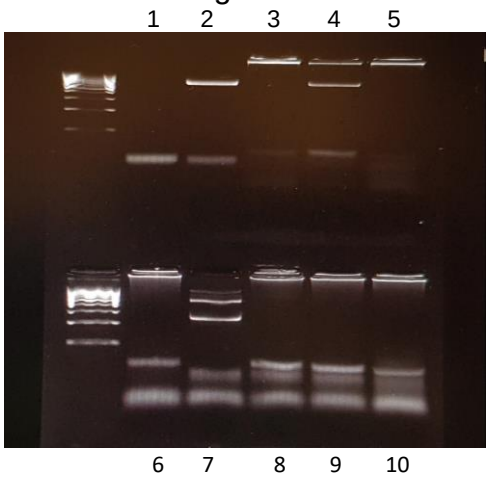
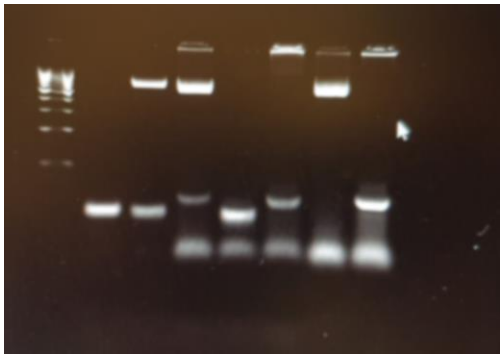


Fig. 25b



1 2 11 12 13 14 15

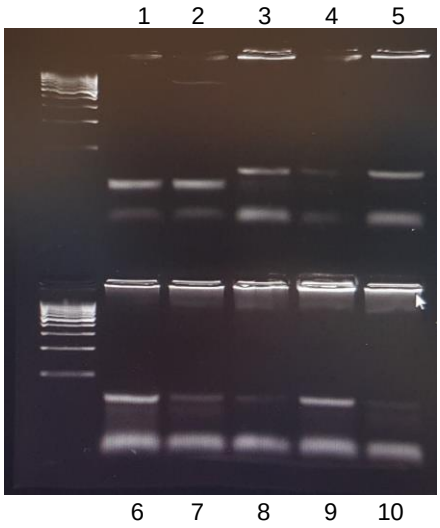
The results are interesting. An amplicon of ca. 945 bp was detected for the control sample No. 4 (C-1, 1-16-2020 ♂), which migrated the same distance as the V23B virus amplicon. Does this mean that there is a latent or covert virus infection present in the wild CRB-G population? [**Covert** infection can comprise either non-productive latency or sublethal infection involving low level production of **virus** progeny]. Also, in virus treated sample No. 11, which was an intentionally V23B infected beetle as part of the autodissemination study (T-12 IM/UF 1/19/2020 ♂), an amplicon was detected that was somewhat smaller than the expected 945 bp. Could this be a variant of the V23B isolate or something else entirely?
This same size amplicon was also detected in sample No. 14 (T-5 IM/UF 1/19/2020 ♂), but this was expected since the male was treated with the virus. However, the female exposed to this oral infected-treated male in the same bottle showed no such amplification when subjected to PCR analysis (sample No.8, T-5 IM/UF 1/19/2020 ♀), indicating an absence of virus transmission. DNA sequencing of all these fragments will be required to confirm any virus transmission.

PCR amplification results of 1-31-2020 in Table 26 was performed employing the following running conditions and **15a/15b** primers: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 50^0C for 45 s, and 72^0C for 1min; and (3) a final extension at 72^0C for 5 min. Samples tested were from an autodissemination study to determine the potential transmission of virus by laboratory infected adult beetles to non-infected adults. 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 2% Metaphor gel (TBE buffer, pH 8.3) with a Beijing Liuyi DYY-6C power supply at a constant 120 v. V23B virus isolate generated the expected DNA amplicon size (945 bp). The gel was stained with Gel Green (Caroline Biological Supply) and photographed using a BioRad GelDoc XR+ imager.

Table 26

Primer set	Sample No.	Sample description	expected fragment (bp)
15a/15b	1	negative control	-
"	2	V23B virus cell culture ➡	**945**
"	3	C-4 1/21/2020 ♂	-
	4	C-23 F/M 1/21//2020 ♂	-
"	5	C-12 1/21/2020 ♀	-
"	6	C-18 M/F 1/23/2020 ♂	-
"	7	T-4 IF/UM 1/27/2020 ♀	-
"	8	T-6 IM/UF 1/25/2020 ♀	-
"	9	T-6 IF/UM 1/21/2020 ♂	-
"	10	T-2 IM/UF 1/27/2020 ♀	-

Fig. 26



PCR amplification results of samples taken from the autodissemination test. Non-treated controls and virus-treated beetles are included on the gel. The only specific band amplified though faint was the V23B DNA positive control.

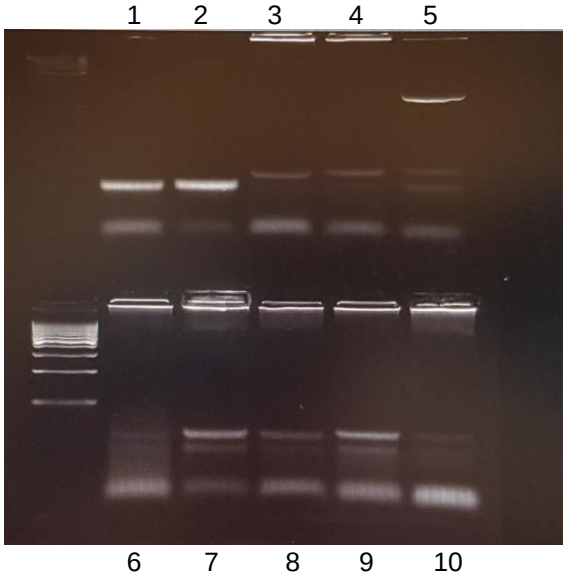
PCR amplification results of 2-3-2020 in Table 27 was performed employing the following running conditions and **15a/15b** primers: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 50^0C for 45 s, and 72^0C for 1min; and (3) a final extension at 72^0C for 5 min. Samples tested were from an autodissemination study to determine the potential transmission of virus by laboratory infected adult beetles to non-infected adults. 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 2% Metaphor gel (TBE buffer, pH 8.3) with a Beijing Liuyi DYY-6C power supply at a constant 120 v. V23B virus isolate generated the expected DNA amplicon size (945 bp). The gel was stained with Gel Green (Caroline Biological Supply) and photographed using a BioRad GelDoc XR+ imager.

Table 27

Primer set	Sample No.	Sample description	expected fragment (bp)
Adv-F1/R1	1	negative control ➡	-
"	2	V23B virus cell culture	**ca. 945**
"	3	C-11 1/25/2020 ♂	< 100
	4	C-9 1/25//2020 ♀	< 100
"	5	C-24 F/M 1/23/2020 ♀	< 300
"	6	C-8 1/23/2020 ♀	< 100
"	7	C-17 M/F 1/21/2020 ♀	< 100
"	8	C-8 1/23/2020 ♂	< 100
"	9	T-6 IF/UM 1/21/2020 ♂	< 100
"	10	T-2 IM/UF 1/27/2020 ♀	< 100

Fig. 27



The expected band for the positive control as well as the other test samples were not amplified during this run. So even if the expected DNA amplified products were detected for the test samples, the whole PCR run would be problematic without an identifiable positive control band. There were besides an excess of primers displayed at the bottom

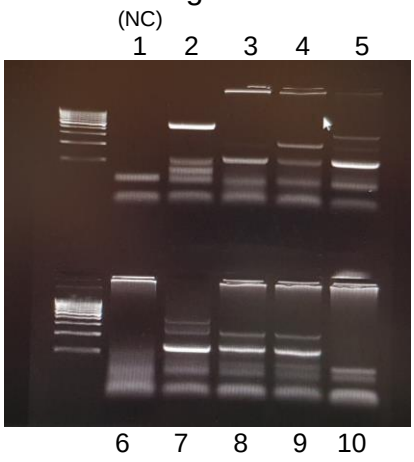
of the gel, other smaller unknown amplified DNA fragments present on the gel just above the primers.

PCR amplification results of 2-4-2020 in Table 28 was performed employing the following running conditions and **AdV-F1/R1** primers: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 57^0C for 45 s, and 72^0C for 45 s; and (3) a final extension at 72^0C for 5 min. Samples tested were from an autodissemination study to determine the potential transmission of virus by laboratory infected adult beetles to non-infected adults. 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. V23B virus isolate generated the expected DNA amplicon size (945 bp). The gel was stained with Gel Green (Caroline Biological Supply) and photographed using a BioRad GelDoc XR+ imager.

Table 28

Primer set	Sample	Sample description	Exp. fragment (bp)
Adv-F1/R1	1	negative control ➡	-
"	2	V23B virus cell culture	**644**
"	3	C-11 1/25/2020 ♂	< 200
	4	C-9 1/25//2020 ♀	< 200
"	5	C-24 F/M 1/23/2020 ♀	< 200
"	6	C-8 1/23/2020 ♀	< 300
"	7	C-17 M/F 1/21/2020 ♀	< 200
"	8	C-8 1/23/2020 ♂	< 200
"	9	T-6 IF/UM 1/21/2020 ♂	< 200
"	10	T-2 IM/UF 1/27/2020 ♀	< 100

Fig. 28



The PCR run amplified the expected product for the positive control but the test samples showed no product amplification. However, the test samples did display smaller non-specific band fragments between 200-300 bp in length.

PCR amplification results of 2-10-2020 in Table 29 was performed employing the following running conditions and **15A/15b** primers: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 57^0C for 45 s, and 72^0C for 45 s; and (3) a final extension at 72^0C for 5 min. Samples tested were from an autodissemination study to determine the potential transmission of virus by laboratory infected adult beetles to non-infected adults. 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.

Table 29

Primer set	Sample	Sample description	Exp. fragment (bp)
15 a/15b	1	negative control	-
"	2	V23B cell-culture virus DNA	-
"	3	Dug42 cell-culture virus DNA	945
"	4	S2A cell-culture virus DNA	945
"	5	PNG cell-culture virus DNA	945

PCR amplification results of 2-10-2020 in Table 30 was performed employing the following running conditions and **AdV-F1/R1** primers: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 57^0C for 45 s, and 72^0C for 45 s; and (3) a final extension at 72^0C for 5 min. Samples tested were from an autodissemination study to determine the potential transmission of virus by laboratory infected adult beetles to non-infected adults. 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.

Table 30

Primer set	Sample	Sample description	Exp. fragment (bp)
AdV-F1/R1	6	negative control	-
“	7	V23B cell-culture virus DNA	644
“	8	Dug42 cell-culture virus DNA	644
“	9	S2A cell-culture virus DNA	644
“	10	PNG cell-culture virus DNA	644

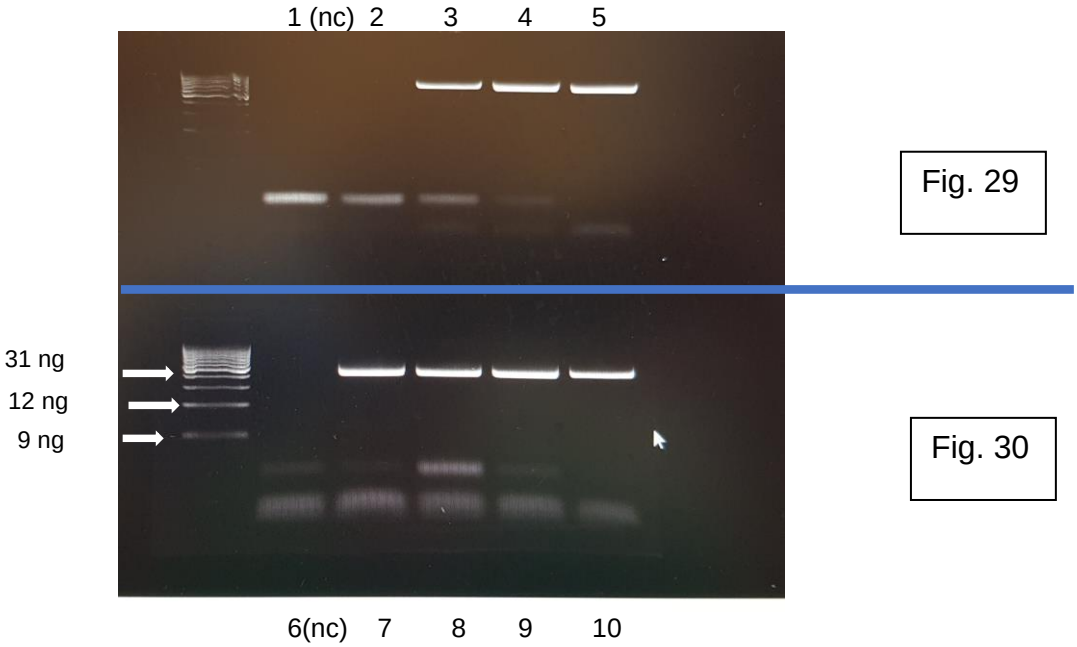
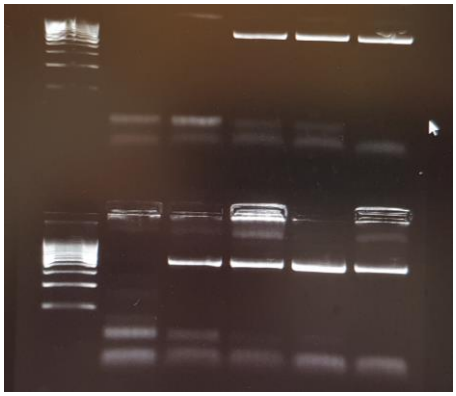


Fig. 29

Fig. 30

The above photograph shows the results for all the positive control samples generated from the following four OrNV isolates: V23B, Dug42, S2A, and PNG. The only amplicon product that was not amplified was from the V23B isolate employing the 15a/15b primers.

Gel photograph of PCR analysis of samples sent to Genewiz for Sanger sequencing employing the 15a/15b primers. This is a second PCR run of samples depicted in Figs. 29, 30.

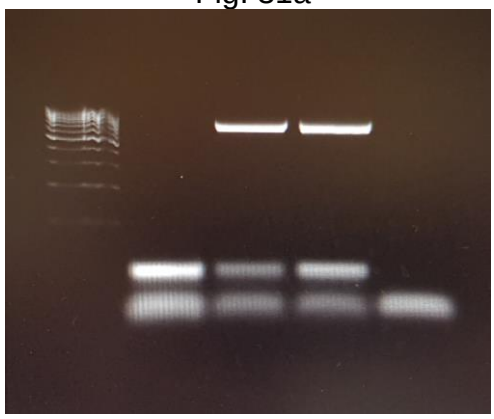


PCR amplification results of 2-10-2020 in Table 31 was performed employing the following running conditions and **15A/15b** primers: (1) Denaturation at 94^0C for 30 s; (2) 35 cycles of 94^0C for 30 s, 57^0C for 45 s, and 72^0C for 45 s; and (3) a final extension at 72^0C for 5 min. This run was to see if just using the Promega DNA extraction kit would yield a sufficient amount of virus DNA without having to employ the BioVision extraction kit with its magnetic beads (lose sample with these beads).
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.

Table 31

Primer set	Sample	Sample description	Exp. fragment (bp)
15a/15b	1	negative control	-
"	2	V23B cell-culture virus DNA DNA extraction using Promega	700
"	3	S2A cell-culture virus DNA DNA extraction using Promega	700
"	4	V23B cell-culture virus DNA DNA extraction (BioVision beads)	700

Fig. 31a



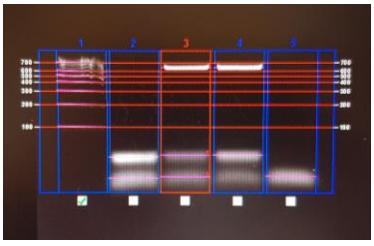


Fig. 31b

Based on the standard DNA marker used in this amplification, the PCR run produced DNA fragments smaller than expected from both V23B and S2A virus isolates, whose DNA was isolated via a Promega DNA extraction kit. The same smaller size fragment was also detected (too faint to see in the above photograph) for the V23B isolate whose

DNA was isolated from a BioVision DNA extraction kit. The commercial DNA marker used in this and in many previous PCR runs have been poorly delineated on the gels; consequently, it is difficult to be certain as to the actual bp size of the amplified unknown fragment.

The latest DNA sequences were received from Genewiz (2/25/2020) and 2 of the 7 were really good. The 2 (PNG and S2A isolates) showed that what we call "positive escontrols" are indeed positive controls. I ran V23B and Dug42 which did not sequence. It was a problem with the priming reaction. I just need to readjust the primer concentrations and rerun them again, but I at least feel confident we have good positive controls.

QS- (Quality Score) - The average of the Quality Values (QV) for each base in the sequence. The QV is derived from this formula [QV = - 10log10(Pe) where Pe is the Probability of Error]. QV 1-10 (Pe of 79%- 10%), QV 11-20 (Pe of 7.9%-1.0%), QV 21-30 (Pe of 0.79%-0.10%), QV 31-40 (Pe of 0.079%-0.010%), QV 50 or more (Pe of 0.001% or less). CRL (Contiguous Read Length) - This is the longest uninterrupted stretch of bases with quality value (QV) higher than 20 within a specified window. This means that the adjacent bases are also considered when calculating this value. A CRL value of 500 or more passes our quality control