DETECTION OF *Oryctes rhinoceros* NUDIVIRUS (ORNV) FROM *Oryctes rhinoceros* (COLEOPTERA: SCARABAEIDAE) BEETLES IN OIL PALM PLANTATIONS OF EAST COAST PENINSULAR MALAYSIA

Puvarasi Pallanisamay[1], Siti Nor Khadijah Addis[1], Nur Ain Farhah Ros Saidon Khudri[2], Ramle Moslim[2] and Wahizatul Afzan Azmi[1,3*]

[1] Faculty of Science and Marine Environment, Universiti Malaysia Terengganu,
21030, Kuala Nerus, Terengganu, Malaysia.
[2] Malaysian Palm Oil Board (MPOB), 6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.
[3] Institute of Tropical Biodiversity and Sustainable Development (ITBSD),
Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia.
*Corresponding author: wahizatul@umt.edu.my

ABSTRACT

Rhinoceros beetle, *Oryctes rhinoceros* (L.) is an economically important pest of oil palm in Southeast Asia and few management practices were carried out to manage the population. The use of a biological control agent such as *O. rhinoceros* nudivirus (OrNV) is the prefered way to manage this pest. This research sought to detect OrNV from the guts of *O. rhinoceros* beetle adults and determine the strain of the virus in selected oil palm plantations due to the scarcity of information available mainly in the east coast of Peninsular Malaysia. The captured rhinoceros beetles were examined for the presence of the virus by polymerase chain reaction (PCR) detection method using *Hind*III to determine the strain of the OrNV. All samples were positive for OrNV. DNA genomic profiles produced revealed that the strain type was OrNV Type A for Terengganu and Kelantan samples. Type A is considered less virulent and has low efficiency in inhibiting the population size of rhinoceros beetles as compared with Type B of OrNV. The information obtained from this study serves as a basis for a better understanding on the occurrence of OrNV primarily in the east coast of Peninsular Malaysia which will help identify the virus type in a particular location before the pre-release site assessment of OrNV against the rhinoceros beetles.

Key words: biological control, pathogenic virus, sustainable agriculture, integrated pest management.

INTRODUCTION

Malaysia became the world's largest palm oil producer in 1999, producing 10.6 million metric tons, which is more than half (51%) of the total global production (Palm Oil World, 2011). Around 20 years later, 18.5 million metric tons of palm oil is exported in 2019 (Malaysia Palm Oil Council, 2020) following the conversion of 5,230 hectares of land into oil palm plantations in the Malaysian peninsula (Gunarso et al. 2013).

The outbreaks of the rhinoceros beetle, *Oryctes rhinoceros* Linnaeus (Coleoptera; Scarabaeidae) have greatly affected the sustainability of the oil palm development in both Malaysia and Southeast Asia (Zelazny and Alfiler 1986; Rahayuwati et al. 2020). Since a preclusion on smouldering for clearance of old palms and organic matter was presented in the1990's, it became a serious pest (Ramle et al. 2005). Rhinoceros beetle adults are troublesome during the formation of

young palms and harm is relied upon to increment with the current broad replanting plans in Malaysia, which give a wealth of reproducing sites for the pest and substantial quantities of vulnerable young palms. Rhinoceros beetle attacks on the oil palm cause fronds to be reduced by 15%. This leads to reduced photosynthetic activity, resulting in delayed maturity of the plant and a decrease of the fruit bunch size. Such phenomena affect oil palm production and reduce profit for Malaysia (May 2012).
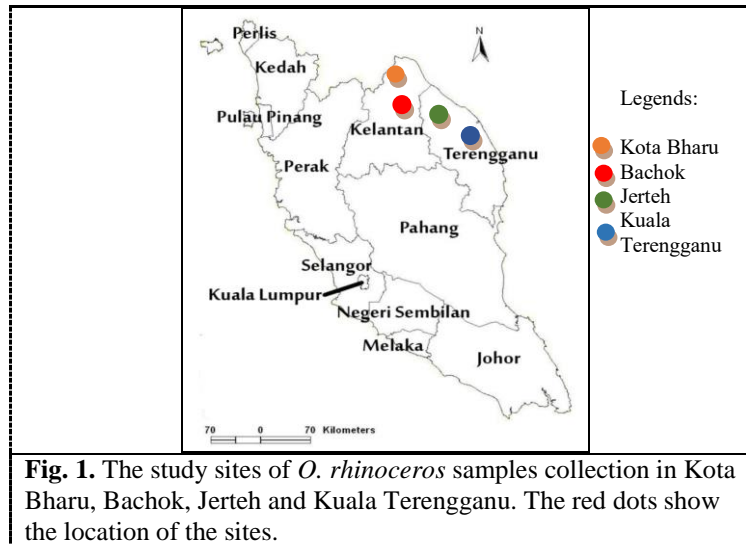
Few current practices have been carried out to control the rhinoceros beetle population of which biological control is one of the preferable approaches as it is safe to non-living organisms (Bedford 2013a; 2014). Therefore, *O. rhinoceros* Nudivirus (OrNV) is preferable as a biocontrol agent since it is highly host specific and non-toxic to the environment (Bedford 2013b; 2014). OrNV acts as a natural enemy by reducing the establishment of new breeding sites and emergence of a new generation. The infection of OrNV is peroral and it replicates in the midgut of adults and midgut and fat body of larvae (Bedford 2013b; Marshall et al. 2017). OrNV replicates in the nucleus of the infected midgut and fat body cells which in turn shortens the life range and fertility. Infected adults become the vector by defecation and mating with other adults, where it disperses the virus and taint the larvae and other adults. The establishment of non-endemic OrNV in Andaman, Maldives, Minicoy and Oman islands showed reduction in population of beetles and damages to palm (Bedford 2013b). The successful establishment of the virus and embodiment into integrated pest measures were recorded in many regions (Huger 2005).

Although the OrNV was first discovered in Malaysia about 40 years ago, limited research has been carried out. There are genetic variations in different types of the virus and the endonuclease enzyme, *Hind*III has been used to characterise the OrNV from different regions in Asia (Crawford et al. 1985). Since there are four types of OrNV namely A, B, C and D, the management strategy should emphasize on the virulence as different strain have shown different effectiveness in killing the rhinoceros beetles. The OrNV Type B is the most pathogenic against the rhinoceros beetle and has the potential to be used for the integrated pest management of this pest (Ramle et al. 2011). From an environmental point of view the virulence is characterized as bringing quick destruction (Bedford 2013b) and helps to increase rhinoceros beetle mortality, which in turn increase the productivity of palm oil (Ramle et al. 2005; Nur Ain Farhah et al. 2016).

To fully utilize the OrNV for better control of *O. rhinoceros,* it is crucial to identify the type of the virus in the area before a pre-release site assessment is done (Ramle et al. 2011). However, there is little information available on the natural occurrence of the indigenous OrNV particularly in Terengganu and Kelantan. Therefore, this study sought to determine the strain type of OrNV from the guts of adult *O. rhinoceros* beetles in selected oil palm plantations of the East Coast of Peninsular Malaysia. This study is important to gain a better understanding of the types of OrNV from various locations in Malaysia that can be potentially developed as a good biological control agent of the rhinoceros beetles.

MATERIALS AND METHODS

Sample collection. *Oryctes rhinoceros* beetles were collected using the pheromone trapping method based on Norman et al. (2007) with minor modifications. There were four sites of oil palm plantations in the East Coast of Peninsular Malaysia that were selected for sample collections. Jerteh and Kuala Terengganu were selected as sites for trapping in Terengganu, while Kota Bharu and Bachok were the selected sites for trapping in Kelantan (Fig. 1). The sites were selected as oil palm trees in the areas exhibited signs of infestation by the *O. rhinoceros* beetles. Besides, previous studies did not cover the selected sites (Ramle et al. 2005; 2011) and limited information is available on OrNV distribution in the east coast of Peninsular Malaysia.

Fig. 1. The study sites of *O. rhinoceros* samples collection in Kota Bharu, Bachok, Jerteh and Kuala Terengganu. The red dots show the location of the sites.

Sample preparation. Equipment were sterilized before use by soaking in 90% alcohol or autoclaving. Bench surface and equipment were wiped with 0.1M HCl to deactivate any DNA and 70% of ethanol was used to remove any residue or microbial contamination. The head and pronotum of the beetles were removed by using scissors, the internal organs were pulled apart and the entire gut tissue was removed using forceps and then divided into two portions. The first portion of 1 cm long gut was placed into a microtube with 150µl of Tris HCl pH 7.5 for the next analysis. The second portion of the rest gut tissue was placed into a new microtube with 500µl Tris HCl pH7.5 for storage purpose (Ramle et al. 2005; Nur Ain Farhah et al. 2016).

DNA extraction. The 1 cm of gut tissue stored in 150µl of Tris HCl pH 7.5 was homogenised until the gut was fully dissolved. The tissue was spun at 13,000 rpm for 2 mins. About 150 µl of supernatant was transferred to a new a 1.5ml centrifuge tube. Disruption buffer (300µl) consisting of 50µl of 1.0mM Tris pH8 + 232.5µl of $sMqH_2O$ + 10µl 1.0 M EDTA + 2.5µl Proteinase K (20 mg ml^{-1}) + 5µl 10% SDS was added and mixed until the solution turned cloudy. The solution was incubated in a waterbath at 65°C for 1 h. An equal volume of phenol; chloroform; isoamylalcohol (PCI) (25; 24; 1) were added and the solution was mixed by inverting the tube for 100 times. The tube was spun again at 13 000 rpm for 10 mins. About 400-500 µl of the upper layer was transferred to a new 1.5ml centrifuge tube. 50µl of 3M NaAC pH8 and 900µl of alcohol were added and stored in -20°C for 1 h. The solution was spun at 13 000rpm for 10 mins and the solution was decanted and discarded. The DNA pellet was dried at room temperature in a biohazard cabinet. 100µl of TE buffer (10mM Tris HCl pH7.5, 1mM EDTA pH8) was added and the solution was dissolved using vortex (Ramle et al. 2005).

Polymerase chain reaction (PCR). Approximately 22µl PCR mix solution containing 14.63µl of SMqH2O, 1.25µl of MgCl2(Promega), 2.5µl of 10X PCR buffer (10mM), 0.5µl DNTP (Promega), 1µl of primer 15A (5' ATTACGTCGTAGAGGCAATC 3') (IDT), 1µl of primer 15B 5' CATGATCGATTCGTCTATGG 3') (IDT) (Richards et al. 1999), 0.125µl *Taq* DNA polymerase (Promega) and PCR mix solution and 1µl BSA (20mg/ml) were prepared. 4µl of DNA solution was added into the PCR mix solution in a 0.2ml PCR tube. For positive control, 4µl of total DNA extract of *O.rhinoceros* infected by OrNV was added into the PCR mix and was mixed gently. For negative control, 4µl of SMqH2O was added into the PCR mix. The PCR was run for 30 cycles; denaturing at 94°C for 1 minute; annealing at 50°C for 2 mins and extension at 72°C for 1 minute (Ramle et al. 2005; Nur Ain Farhah et al. 2016).

PCR product visualization. One percent of the agarose gel was prepared to run the PCR product in 60ml casting tray. Agarose powder (0.6g) was mixed with 60ml 1X TAE (Tris-Acetate-Ethylenediamine tetraacetic acid) solution. The agarose solution was heated for several mins in a microwave oven until the powder dissolved fully in 1X TAE solution. The solution was cooled down to around 60 °C. 1.5µl of ethidium bromide was added to the gel solution. The gel was poured slowly into the casting tray with well comb in place and the gel was left to solidify for 30 mins at room temperature. 10µl of loading buffer was added into each PCR tube. For DNA marker, 3µl of 1Kbp DNA ladder (Promega) was mixed with 10µl loading buffer. The tray was then connected with power supply and ran at power 85-150V until the dye line is about 75-80% down the gel. The time to complete the gel electrophoresis depends on the voltage and concentration of gel. DNA fragments or bands were visualized under UV light. The 1 Kbp DNA ladder was used in the first lane to analyse the bands produced (Ramle et al. 2005).

Restriction Enzyme Analysis by *Hind*III enzymes

Genomic Extraction of DNA. Homogenised infected guts which confirmed as positive for virus by PCR were grouped and transferred in 1000µl Tris HCl, pH7.5 and spun at 13,000 rpm for 2 mins. The supernatant (1ml) was transferred to new tube and filtered through 0.45µl filter membrane. The solution was transferred to a new 12 ml tube and the tubes were ensured of the same final weight. The solution was spun at 30,000 rpm for 2 h at 10°C. The solution was then decanted and discarded. 600µl of disruption buffer (double recipe of disruption buffer in DNA extraction) was added and homogenised until the DNA pellet was fully dissolved. The solution was transferred to 2ml centrifuge tube and later incubated in a waterbath at 65°C for an hour. Equal volume of PCI was added to the solution and the tube was inverted for 100 times. The solution was spun at 13,000rpm for 10 min. and the upper solution was transferred to a new tube. 1µl RNAse (20mg/ml) was added and incubated again in waterbath at 65°C for 1 h. Equal volume of PCI was added again to the solution and tube was inverted for 100 times. Then, the solution spun at 13,000rpm for 10 mins and the upper layer was transferred to a new tube. About 60 µl of 3M NaAc and 1,200 µl of 100% alcohol have been added to the solution and placed in -30°C for 1 h. The solution was centrifuged again at 13,000 rpm for 15 min., decanted and discarded. The DNA pellet was dried at room temperature in a biohazard cabinet. About 35µl of TE buffer was added and shaken to dissolve the pellet. Then, 2µl of genomic sample was mixed with 10µl loading buffer and ran on 0.8% agarose gel for DNA quality check (Ramle et al. 2005; Nur Ain Farhah et al. 2016).

Restriction Enzyme Digestion. The mixed solution of Hind*III* enzyme and DNA was incubated in a waterbath at 37°C for 6 h. Then, 60 µl of 3M NaAc and 1,200 µl of 100% alcohol were added to the solution and incubated again at 4°C for 30 mins. The solution was centrifuged at 13,000rpm for 15 mins. The supernatant was discarded and the pellet was dried in biohazard cabinet. The pellet was suspended again in 10µl Tris buffer (pH8) and about 2µl of loading buffer was added to the digested genomic sample and ran on 0.8% of agarose gel at 35V for 20 h. (Ramle et al. 2005).

RESULTS AND DISCUSSION

Screening of *Oryctes rhinoceros* nudivirus. A total of 32 individuals of *O. rhinoceros* were confirmed infected with *O. rhinoceros* nudivirus (OrNV) in the gut of the beetles from the sampling sites. The total number of beetles captured from each site and the percentage of OrNV in the captured beetles (Table 1). All the *O. rhinoceros* beetles that were collected from four sampling sites were confirmed with the presence of OrNV. The banding pattern appearance of DNA fragments of the beetle's gut was confirmed by PCR as described in Fig. 2 (Jerteh and Kuala Terengganu samples), Fig. 3 (Bachok samples) and Fig. 4 (Kota Bharu samples).

Table 1. The total number of captured *O. rhinoceros* beetles from each study site and percentage of OrNV infection the samples collected.

Collection sites	Total number of captured beetles	Percentage of OrNV infection (%)
Jerteh	9	100
Kuala Terengganu	5	100
Kota Bharu	12	100
Bachok	6	100
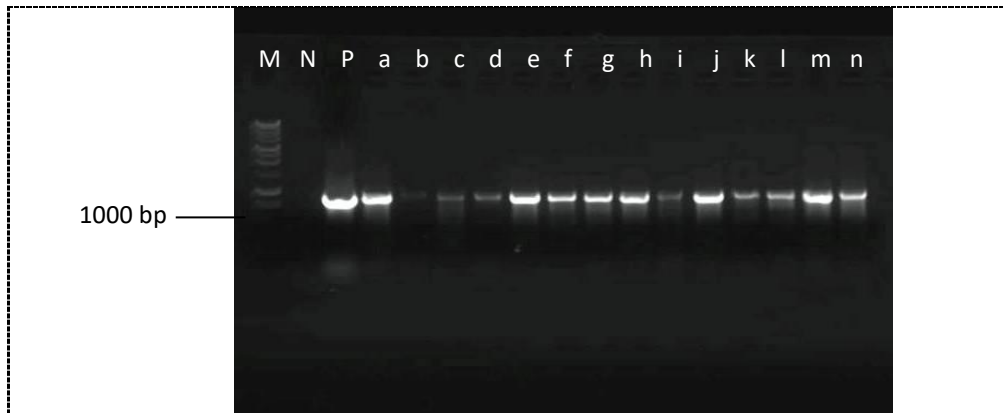
Fig. 2. Banding pattern appearance of samples taken from Jerteh and Kuala Terengganu, Terengganu showing the appearance of a single band at size 945 bp which confirmed the presence of *Oryctes rhinoceros* nudivirus. Note that M = Marker (1 Kb DNA ladder), N = Negative control, P = Positive control, a- i = Jerteh samples, j-n = Kuala Terengganu samples.
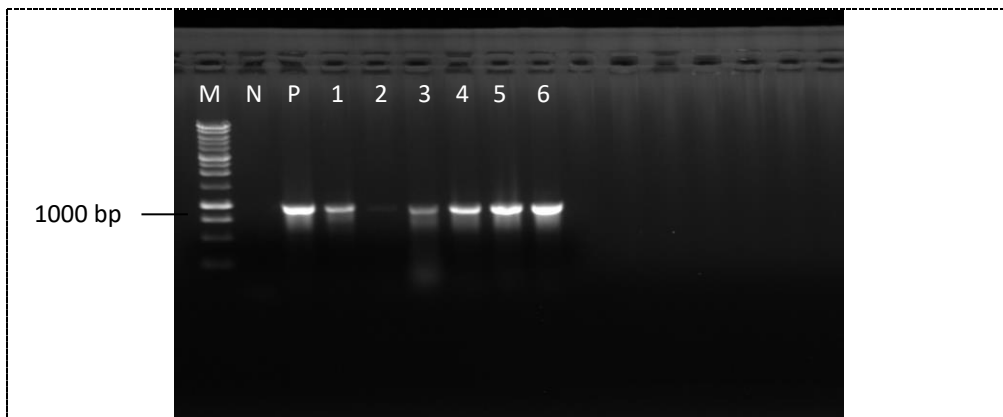
Fig. 3. Banding pattern appearance of samples taken from Bachok, Kelantan showing the appearance of a single band at size 945 bp which confirmed the presence of *Oryctes rhinoceros* nudivirus. Note that M = Marker (1 Kb DNA ladder), N = Negative control, P = Positive control, 1-6 = Bachok samples.
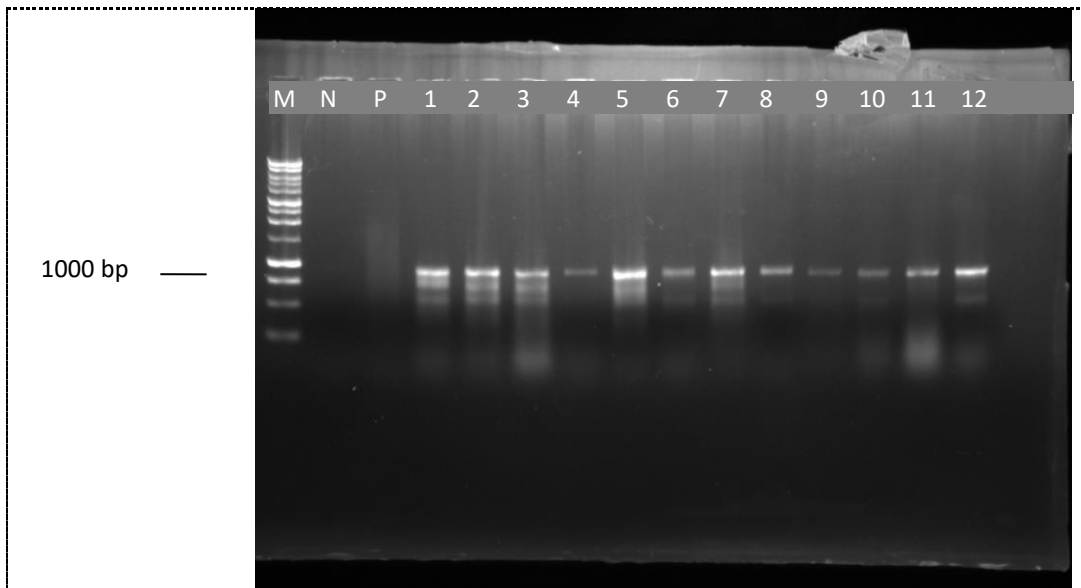
Fig. 4. Banding pattern appearance of samples taken from Kota Bharu, Kelantan showing the appearance of a single band at size 945 bp which confirmed the presence of *Oryctes rhinoceros* nudivirus. Note that M = Marker (1 Kb DNA ladder), N = Negative control, P = Positive control, 1-12 = Kota Bharu samples.

This study revealed that all the samples obtained from the four sites showed the presence of OrNV. Numerous previous studies have shown that the incidence of OrNV is widespread in Malaysia (Manjeri et al. 2014). The presence of OrNV during the dissection of the beetles could be noted by the appearance of swollen guts, a typical symptom of the infected gut tissues by the OrNV (Kumar and Ahmad 2008; Marshall et al. 2017).

Identification of viral types. *Oryctes rhinoceros* collected from Kuala Terengganu showed low virus yield. Samples collected from Bachok and Kota Bharu showed high yield with clear DNA banding pattern, while samples from Jerteh showed no yield with no visible DNA fragment during the DNA quality check. The DNA profiles produced after digestion of *Hind*III enzyme for Kuala Terengganu, Bachok and Kota Bharu samples and were confirmed to be Type A of OrNV according to the DNA bands (Ramle et al. 2005) (Fig. 5). The insertion of a single band at different size differentiates the strain types. Type B has a single insertion band of size 15,471bp and Type C has an insertion at size 2.0 kbp. Type D has an insertion of two DNA bands at 15,471bp and also between 2.0 and 1.5kbp (Ramle et al. 2011). Thus, the absence of insertion of the single band at size 15,471bp, 2.0 kbp and between 2.0 and 1.5 kbp indicated the samples as Type A. The presence of a band at 945 bp on each sample was a clear indication that the cells were infected by OrNV Type A (Fig. 2, 3 and 4).

Quantification of DNA is necessary to determine the amount of DNA that is present prior to restriction enzyme analysis (Murali and Alka 2002). Jerteh samples showed no yield of virus compared to the other three sites which may be due to technical error. The other three samples showed better yield appearance. The samples were prepared with *Hind*III endonuclease enzyme according to the yield of DNA present in quality check to produce profiles of DNA fragments.
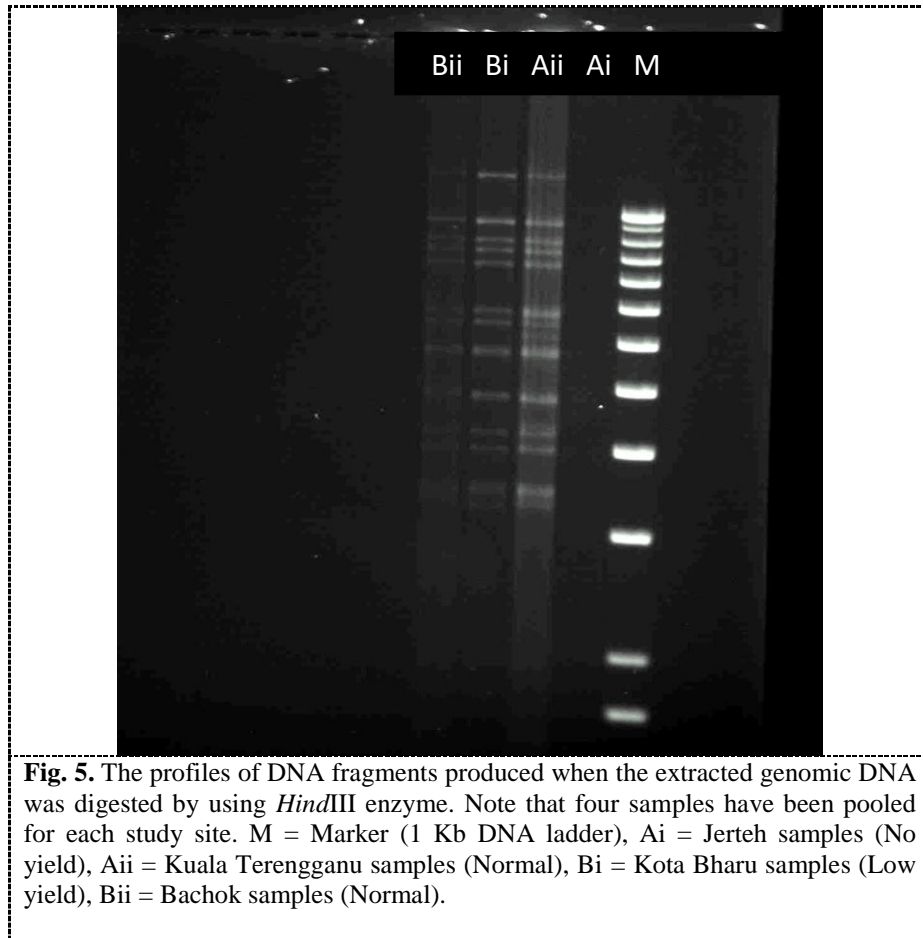
Fig. 5. The profiles of DNA fragments produced when the extracted genomic DNA was digested by using *Hind*III enzyme. Note that four samples have been pooled for each study site. M = Marker (1 Kb DNA ladder), Ai = Jerteh samples (No yield), Aii = Kuala Terengganu samples (Normal), Bi = Kota Bharu samples (Low yield), Bii = Bachok samples (Normal).

Detection of viral types. Based on the profiles of DNA fragments generated after digestion of the *Hind*III endonuclease enzyme, the OrNV Type A virus was identified for samples from Kelantan and Terengganu. The viral type was identified based on reports by Ramlee et al. (2005; 2011). Unfortunately, Jerteh showed no banding pattern, which indicated that there was no yield of virus. This situation proved that the quality check is significant to produce the best profiles of DNA fragments. Although the samples were prepared with high quantity of *Hind*III endonuclease enzyme for Jerteh samples yet there was no band present. Thus, smearing of band during quality check still could be adjusted by increasing the proportion of DNA sample, 10X buffer, enzyme and decreasing SMqH2O to produce the best banding pattern. Zero yields probably indicated low virus particles present in the samples (Ramle et al. 2011).

Four types of OrNV have been revealed: Type A, Type B, Type C and Type D in Malaysia (Ramlee et al. 2005; 2011). Type A virus is common in Peninsular Malaysia and is less virulent compared to Type B which is known as the most virulent and has high efficacy in reducing the number of the beetles. Besides, virus Type C is found in Sabah only and less efficient in causing mortality of larvae and adults (Murali and Alka 2002). Therefore, rhinoceros beetles obtained from the studied sites in Kelantan and Terengganu showed a weak strain of OrNV and are less efficient to control the population of the beetles.

CONCLUSION

The presence of *O. rhinoceros* nudivirus (OrNV) in all the *O. rhinoceros* beetles collected from Jerteh, Kuala Terengganu, Bachok and Kota Bharu was confirmed. The *Hind*III enzyme digestion profiles for Kuala Terengganu, Bachok and Kota Bharu samples were confirmed to be Type A of OrNV. However, Type A is known to be less virulent and has low efficiency in inhibiting the population of rhinoceros beetles. Therefore, the information obtained from this study provides a better understanding of the virus type which will be important in the development of a strategy using a biocontrol agent against rhinoceros beetle before the pre-release site assessment is done. Thus, a necessary measure such as releasing OrNV Type B in the field should be taken to prevent further spread of the beetles. Future research needs to be carried out especially on the field release strategy in oil palm plantations of east coast Peninsular Malaysia.

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