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The incidence and use of *Oryctes* virus for control of rhinoceros beetle in oil palm plantations in Malaysia

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Abstract

The rhinoceros beetle, *Oryctes rhinoceros*, has emerged as a serious pest of oil palm since the prohibition of burning as a method for maintaining estate hygiene in the 1990s. The abundance of beetles is surprising given that the Malay peninsula was the site of first discovery of the *Oryctes* virus, which has been used to effect good as a biological control agent in other regions. A survey of adult beetles was carried out throughout Malaysia using pheromone traps. Captured beetles were examined for presence of virus using both visual/microscopic examination and PCR detection methods. The survey indicated that *Oryctes* virus was common in Malaysia among the adult beetles. Viral DNA analysis was carried out after restriction with *Hin*dIII enzyme and indicated at least three distinct viral genotypes. Bioassays were used to compare the viral strains and demonstrate that one strain (type B) is the most virulent against both larvae and adults of the beetle. Virus type B has been cultured and released into healthy populations where another strain (type A) forms the natural background. Capture and examination of beetles from the release site and surrounding area has shown that the spread and persistence of the applied virus strain is accompanied by a reduction in palm frond damage.

Keywords: *Oryctes* virus; Palm oil; *Oryctes rhinoceros*; Rhinoceros beetle; Biological control

1. Introduction

The rhinoceros beetle, *Oryctes rhinoceros* (L.), has emerged as major pest of oil palm (*Elaeis guineesis*) since a prohibition on burning for clearance of old palms and organic matter was introduced in the 1990s. Rhinoceros beetle adults are particularly troublesome during establishment of young palms and damage is expected to increase with the current extensive replanting schemes in Malaysia, which provide an abundance of breeding sites for the pest as well as large numbers of susceptible young palms. Application of chemical insecticides such as carbofuran and cypermethrin has been widely used, but this method is costly and hazardous to non-target organisms (Chung et al., 1991). As an alternative, Malaysian Palm Oil Board (MPOB) embarked on research for the use of natural enemies such as *Oryctes* virus for control of the pest (Ramle et al., 1999, 2001).

Oryctes virus was discovered in Malaysia about 40 years ago (Huger, 1966) and has since been introduced into many South Pacific and Indian Ocean countries, such as Western Samoa (Marschall, 1970), Mauritius (Hammes, 1978), Tonga (Young and Longworth, 1981), Papua New Guinea (Gorrick, 1980), Philippines (Zelazny and Alfiler, 1991), and Indonesia and Maldives (Zelazny et al., 1992). The virus infects both larvae and adults of rhinoceros beetle and introduction of the virus to Pacific Islands led to a dramatic reduction in pest population and palm damage within 1–2 years (Bedford, 1986).

Although the virus was first discovered on the Malay peninsular, limited research has been conducted to fully

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exploit the potential use of the virus for *Oryctes* control in Malaysia (Ho, 1996). This paper reports on recent initiatives to determine the incidence and variability of *Oryctes* virus in Malaysia and research on methods to use *Oryctes* virus for control of rhinoceros beetle in Malaysian oil palm plantations.

2. Materials and methods

2.1. Incidence of Oryctes virus infection among adult beetles

A survey to determine the incidence of *Oryctes* virus in adult rhinoceros beetles was conducted in 1999–2000. Adults were collected from various oil palm plantations using traps baited with the attractant ethyl 4-methyloctanoate (Chung, 1997) and sent to MPOB's laboratory for virus detection. To ensure high survival during transportation, samples of adults were initially grouped and placed in a closed container containing sugarcane as a food source. In later samples, beetles were held in individual tubes and collected from breeding sites as well as traps. In the laboratory, adults were dissected and guts were collected for DNA extraction. Visual appearance of the guts was recorded. Detection of virus was then performed using the polymerase chain reaction (PCR) method (Ramle et al., 2001) with primers developed by (Richards et al., 1999). The PCR products were run on 2% agarose gel prepared in 1× TAE, stained in ethidium bromide, and photographed using Polaroid film no. 665. The presence of virus was confirmed when amplified DNA fragments at size 945 bp appeared on the agarose gel.

2.2. Incidence of Oryctes virus among larvae

The incidence of *Oryctes* virus on first (L1), second (L2), and third instar larvae (L3) of rhinoceros beetles was determined in samples collected from two sites, Jendarata Estate in Perak and Sing Mah Estate in Johor. Midgut tissues were dissected from the larvae, the gut contents were removed and a section of tissue (approx. 5 mm) was isolated and washed with distilled water. DNA was extracted from the gut tissues and the presence of virus was confirmed as described above.

2.3. Identification of virus type

Viral DNA was extracted from adults with guts showing advanced symptoms of infection. Gut tissues were homogenized in 1.5 ml tubes containing sterilized distilled water and then centrifuged at 13,000 rpm for 5 min to remove cell debris. The supernatant was filtered through a membrane filter, pore size of 0.45 μm, into a 12.5 ml centrifuge tube. The samples were then spun at 130,000 rpm for 2 h to sediment the virus particles. Extraction of virus DNA was performed by adding of 600 μl disruption buffer containing 50 μl 1 M Tris, pH 8.0, 10 μl 0.5 M EDTA, 5 μl 10% SDS, 2.5 μl Proteinase K (20 mg/ml), and 232.5 μl sterilized distilled water. The mixture was incubated at 65 °C for 24 h. An equal volume of phenol–chloroform–isoamylalcohol (25:24:1) was added, then mixed by inverting the tubes 100 times before centrifugation at 13,000 rpm. The aqueous solution was transferred to a fresh tube and the DNA was precipitated by adding 10% NaAc, pH 5.2, and two volumes of absolute ethanol. The DNA was pelleted and suspended in 100 μl TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, pH 8.0).

The volume of DNA solution required for restriction endonuclease digestion was quantified by running 2 μl DNA template on 1.8% agarose. Restriction of virus DNA was performed in 100 μl volume, which contained 10 μl DNA template, 10 μl of 10× buffer, 1.5 μl *Hin*dIII enzymes, and 78.5 μl sterilized distilled water. After incubation for 6 h at 35 °C in a water bath, 10% NaAc, pH 5.2, and two volumes of 100% ethanol were added to the mixture, which was then spun for 5 min to pellet the DNA. The DNA was dried at room temperature and resuspended in 10 μl TE buffer. The viral DNA fragments were electrophoresed on 0.8% agarose gel prepared in 1× TAE and run at 35 V for 16 h.

3. Bioassay to determine the pathogenicity of virus

3.1. Preparation of virus inoculum

The virus inoculum was prepared from homogenized guts of adults collected from Sedenak Estate in Johor (type A), West Estate in Selangor (type B), and Pitas Estate in Sabah (type C). Guts showing swollen symptoms of infection were taken from each of five insects and pooled before maceration in distilled water and centrifugation (as above) to sediment the tissue debris. The homogenate was then filtered through a cellulose nitrate membrane with pore size of 0.45 μm. The suspended virus inoculum was frozen and stored at −20 °C until use. The concentration of virus inoculum for each strain was quantified by comparative PCR before standardization by dilution to a uniform standard.

3.2. Bioassay against larvae

Bioassay of rhinoceros beetle larvae was conducted using mature L3 collected from an Estate in Johor. Inoculation was made by placing 30 μl of virus solution on the mouthparts of the larvae. Inoculated larvae were maintained at 25–28 °C in groups of five in plastic containers with moist oil palm trunk chips. Larvae were examined every 2 weeks for survival over a period of 2

months. All dead larvae were tested for the presence of virus by the PCR method described earlier.

3.3. Bioassay against adults

Late third instar larvae collected from Carey Island Estate in Selangor were reared individually in cylindrical plastic cups (500 ml) to produce adults. Neonate adults were transferred into new containers and kept a week for habituation. Inoculation of adults was carried out by placing 20 µl virus solution prepared in 10% sucrose on the mouthparts of the beetle. Another 10 µl of sucrose solution was placed again on the mouthparts of the beetle to rinse off the remaining virus solution. The number of beetles used in the test was 32 adults for each strain and control, with four replicates. The inoculated adults were placed in cylindrical cages filled with moist oil palm chips. The mortality of adults was monitored weekly. All adult cadavers were tested for the presence of virus.

3.4. Release of virus in the field

A study was conducted at the Malaysia Airport Berhad estate in Selangor, on 6-month-old palms. The area covered about 72 ha of peat soil, ex-cash crop of pumpkin, yam, tapioca, and maize. Decomposing wood and decaying oil palm biomass were abundant on the site. Pre-sampling data showed that the level of virus incidence on adults was 37.5% of type A virus. A total of 150 active adults collected from this area were inoculated with 20 µl type B virus solution prepared in 10% sucrose. The inoculated adults were then re-released into the field by placing five adults on the ground near the base of oil palms in a central zone of about 0.5 ha. Regular pheromone trapping activity at the release site was temporarily halted for 2 weeks, to allow transmission of the released virus type B in the populations. The trapping activity was then continued at a recommended density of 1 trap per 2 ha (Chung, 1997).

The released site was divided into inner, middle, and outer sectors. Pheromone traps were placed in each sector and the distance of each trap from the release point was measured. The virus incidence was estimated from trapped adults. For the first 6 months after release (MAR), adults in each region were collected monthly for 3 consecutive days. Subsequently, collection of adults was made every 3 months until 15 MAR. The number of adults captured in each trap was recorded to estimate the change in population of adults at the release site. Adults were brought back to the laboratory for DNA extraction. In each sampling, only 20% of live adults were used for the viral detection test, the remaining 80% were released back to the field.

In order to measure the impact of the virus release on palm damage, assessment was conducted at 3 and 15 MAR. The proportion of fronds damaged due to rhinoceros beetle attack was estimated. A total of 20 palms were assessed from each region at each time.

4. Results

4.1. Incidence of Oryctes virus

Levels of virus incidence among adult beetles, as detected by PCR, ranged from 0 to 100%, with most site collections indicating >75% of the beetles positive for the virus. The high level of virus indicated by PCR was corroborated by visual examination, where more than 70% of larvae showed the classical milky condition of the gut (Ramle et al., 2001). Visual diagnosis underestimated the true viral incidence because those adults diagnosed as uninfected also had a 70% positive response rate in PCR tests. To account for these very high levels of virus-positive adult beetles, cross-contamination between beetles during trapping or transport from the field to the laboratory was suspected. A detailed study comparing adults caught and transported by different methods (Table 1) showed highest virus incidence from grouped beetles. This was reduced considerably when beetles were held individually suggesting that cross-contamination occurred in the traps and during subsequent transportation. The level of infection was lowest among adult beetles collected from the breeding sites and held individually (Table 1). Infection among larvae was much lower and ranged from 0%, among first and second instar larvae, to 35% among third instar larvae on two sites intensively sampled (data not shown).

4.2. Identification of viral types

Profiles of DNA fragments of virus restricted by *Hin*dIII enzyme are shown in Fig. 1. Three distinct profiles of viral DNA were differentiated. One group, designated type A, produced an identical profile to that produced by Crawford et al. (1985) with strain PV505. Insertion of a single band at approximately 15 kb was used as the identifier of type B. Type C produced a single distinctive band with the size of 2.0 kb. The distribution of viral types from samples collected in Malaysia is presented in

Table 1
Incidence (%) of *Oryctes* virus determined by PCR from adult rhinoceros beetle sampled from three Malaysian estates

	Johore	Pahang	Perak
Container	98.1(54)	94.4(54)	45.9(61)
Individual	65.6(32)	47.2(36)	33.3(15)
Breeding site	52.8(36)	12.5(16)	0(4)

Insects were collected from pheromone traps in groups or as individuals or were collected individually from breeding sites on the same estate. The number of insects tested is given in parentheses.

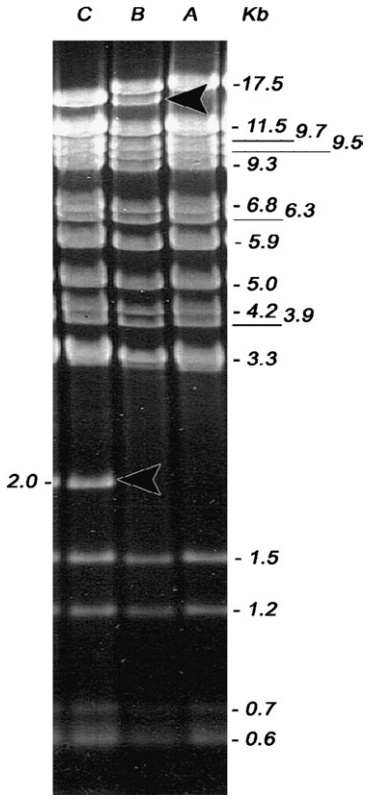

Fig. 1. The profiles of DNA fragments produced when *Oryctes* virus was digested by the *Hin*dIII endonuclease enzyme. Arrows indicate bands that are used to differentiate virus types B and C from A.

Fig. 2. Type A was common and detected in most sites on the Malay peninsular. The presence of type B was restricted to Carey Island in Selangor and Bagan Datuk in Perak. Type C was only detected in West Malaysia, Sabah where neither A or B were detected.

4.3. Pathogenicity tests

Pathogenicity tests indicated that type B caused 70% mortality among larvae, significantly higher than control ($P<0.01$), type A ($P<0.05$) or type C ($P<0.01$). After 2 months from inoculation, type B had caused 86.7% mortality among adults, significantly higher ($P<0.05$) than type A (66.7%) or type C (13.3%). As a consequence of these results, type B was selected for further evaluation in the field.

4.4. Release study of virus in the field

The incidence of virus among collected adults increased from the background level of 35–90% of adults infected by 3 MAR. Restriction analysis indicated that virus type B predominated in the inner sector but was absent from the other sectors and the control area where virus type A was dominant (Fig. 3). From 11 MAR, virus incidence exceeded 90% in all sectors and the control area. Virus type B dominated all sectors and the control plot. The incidence of virus among larvae was tested at 5 and 6 MAR. After this period, no larvae were found, as the breeding materials at the release site were completely decomposed. At 5 MAR, only 7 out of 62 larvae were infected (11.3%), while at 6 MAR, 0 of 6 larvae tested were infected. The results indicate that virus infection had spread throughout the site among adult beetles, but was infrequent among larvae.

5. Discussion

The incidence of *Oryctes* virus recorded from adult beetles collected throughout Malaysia was generally

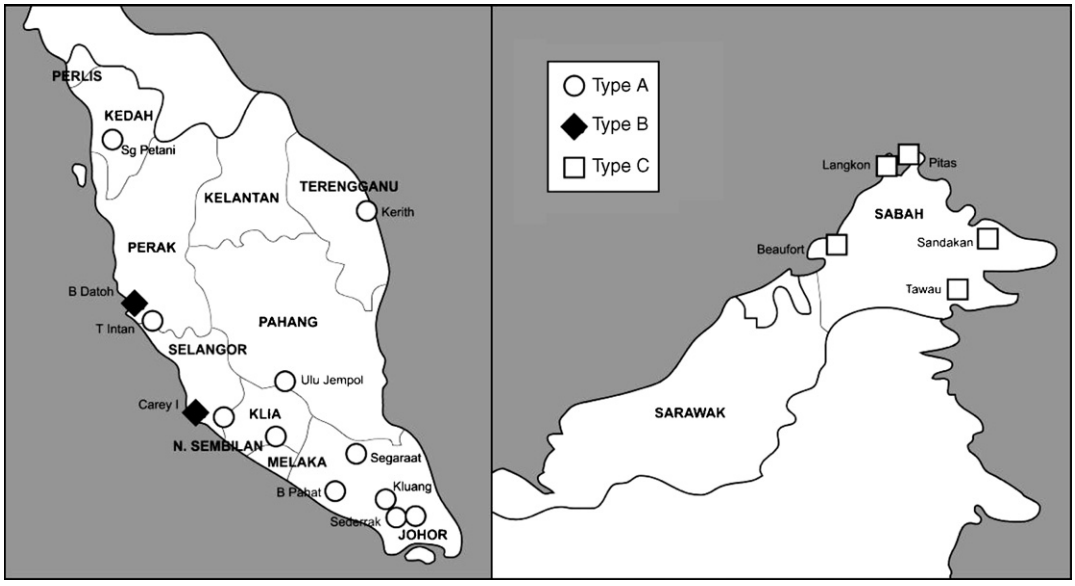

Fig. 2. Geographical distribution of *Oryctes* virus types A, B, and C in Malaysia.

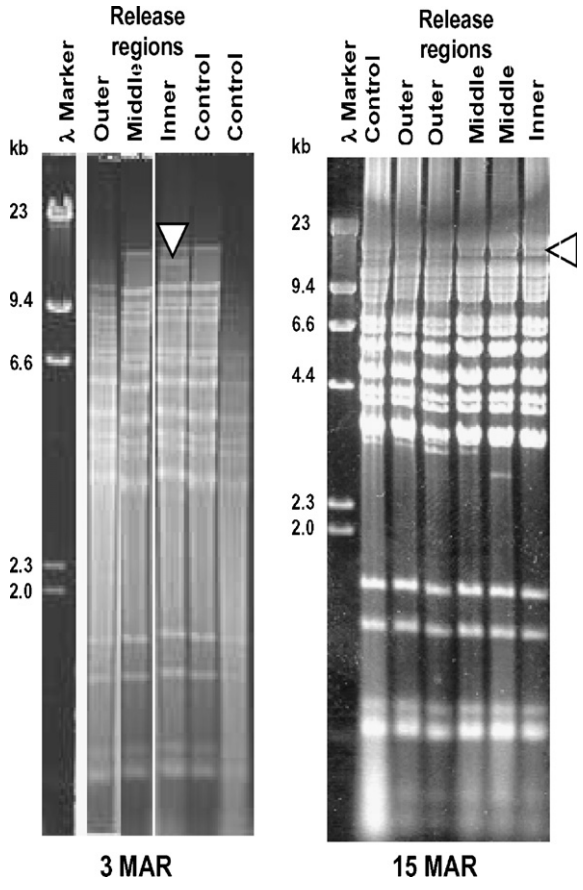

Fig. 3. Oryctes virus restriction profiles for isolates extracted from beetles at the release site at 3 and 15 months after release. Arrows indicate the band that was used to identify the type B virus.

higher than that reported from other countries (Dhileepan, 1994; Zelazny and Alfiler, 1986, 1991; Zelazny et al., 1992), although it did vary between sites. The high levels were probably influenced by two factors. First, the PCR method used was highly sensitive in detecting the virus and could detect virus in apparently healthy larvae before symptoms appeared (Ramle et al., 2001). Second, cross-contamination probably occurred during the transportation of samples from plantations to laboratory leading to an overestimation of the level of disease in the field. It was subsequently recommended that insects be kept individually in vials after capture. However, even when adult beetles were separated and the possibility of cross-contamination minimized, virus incidence still usually exceeded 50% of the sampled beetles.

The incidence of virus among larvae, pupae, and neonate adults in the breeding sites was generally low, suggesting that there is little virus transfer in the rotting organic matter. Adults collected from the breeding sites can be either neonates or returning breeders which could explain the variability in infection levels among this group. When there is an abundance of organic matter, low levels of infection among the larvae mean that the chances of new healthy adults entering the population are high. This could explain why rhinoceros beetle populations can occur at high densities in the Philippines, Indonesia, India, and Malaysia, even where virus disease among the adult beetle population occurs at high levels (Dhileepan, 1994; Zelazny and Alfiler, 1986, 1991).

By genomic analysis, three *Oryctes* viral types were identified from Malaysia. The most abundant, type A, was common throughout the peninsula but showed less efficacy than the restricted genotype B. Type C was only found in Sabah and appeared to have little effect on either larvae or adult beetles. When virus type B was introduced into a high population of rhinoceros beetles with a natural type A virus background it was able to establish and spread among the target beetle population, outcompeting the natural background virus strain.

In conclusion, *Oryctes* virus is widespread in Malaysia and appears to transmit readily within adult beetle populations. Natural viral incidence in larval populations appears to be low, leading to the emergence of large populations of healthy, potentially damaging neonate adults where there are high amounts of rotting organic matter. Localized release of high virulence virus strains has the potential to reduce populations and damage in Malaysian oil palm plantations. The use of virus should be integrated with other control measures, such as release of the fungal pathogen *Metarhizium anisopliae* for control of rhinoceros beetle larvae at breeding sites within the plantations.

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