Journal of Insect Biotechnology and Sericology 79, 117-124 (2010)

Isolation of the Thosea asigna virus (TaV) from the epizootic *Setothosea asigna* larvae collected in South Sumatra and a study on its pathogenicity to Limacodidae larvae in Japan

Maria Sugiharti[1], Chikako Ono[1], Toshiaki Ito[2],
Shin-ichiro Asano[1], Ken Sahara[1], Yulia Pujiastuti[3] and Hisanori Bando[1]*

[1] *Laboratory of Applied Molecular Entomology, Department of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan*
[2] *Electron Microscope Laboratory, Research Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan*
[3] *Department of Plant Pests and Diseases, Faculty of Agriculture, Sriwijaya University, Indonesia*

(Received September 15, 2010; Accepted November 17, 2010)

In 2005, natural epizootics were observed during an outbreak of *Setothosea asigna* (Lepidoptera: Limacodidae) larvae in an oil palm plantation in South Sumatra, Indonesia. The causative agent was filterable, which implied it was a virus. Since preliminary testing using reverse PCR gave a positive result for the Thosea asigna virus (TaV), we experimented with the purification of the viral agent from the diseased larvae. Electron microscopy revealed nonenveloped virus-like particles that were spherical in shape and about 40 nm in diameter. cDNA cloning followed by sequencing demonstrated that RNA purified from the particles contained two large open-reading-frames (ORFs) with a partly shared sequence and extensive homology (> 98% identity at the nucleotide level) with ORFs of TaV encoding an RNA-dependent RNA polymerase and the capsid protein, respectively. 3' RACE suggested that there is, like in TaV genomic RNA, no poly(A) tract at the 3'-terminus of the RNA. The pathogenicity of the purified particles against Limacodidae larvae in Japan was demonstrated to be very strong for *Monema flavescens* and *Austrapoda dentata*. These results indicated that the agent causing the epizootic disease among *S. asigna* larvae in the oil palm plantations was TaV which also has potential as a biological control agent for Limacodidae pests in Japan.

Keyword: Oil palm plantation, Limacodidae, Viruses, Tetraviridae, TaV

INTRODUCTION

The increasing significance of oil palm plantations for the Indonesian economy has raised serious concerns. The plantations regularly use herbicides, insecticides and fertilizers in large amounts. There is a great fear that these agricultural practices cause enough soil- and water- pollution to harm animals and also humans.

S. asigna (Lepidoptera; Limacodidae) is a major defoliating pest of oil and coconuts palms, with outbreaks recorded in north-eastern Sumatra, Borneo and western Malaysia (Holloway *et al*., 1987). Feeding on a wide range of host plants, *S. asigna* has also become an economically important defoliator of oil palms, *Elaeis guineensis*. Their life cycle is about 86-115 days, including a larval stage (1st to 9th instar) of 35-49 days (Godfray *et al*., 1987). In the outbreak, leaf surfaces are rapidly destroyed (up to 50%), resulting in more than a 78% loss of production in the first year and 40% in the second year (Sipayung, 1987).

*To whom correspondence should be addressed.
Laboratory of Applied Molecular Entomology. Graduate School of Agriculture, Hokkaido University, Kita 9 Nishi 9, Sapporo, Hokkaido 060-8589 Japan
Fax & Tel: +81-11-706-3348.
Email: hban@abs.agr.hokudai.ac.jp

In South Sumatra, the first outbreak of *S. asigna*, with a population density of around 13 larvae per frond, was observed in 1999, and continued up to 2002 with more than 25 larvae per frond (Rahmadsyah *et al*., 2003). In 2006, the population of *S. asigna* reached 50 larvae per frond. At this population level, only rib leaves remained on the tree. Furthermore, *S. asigna* can cause caterpillar dermatitis involving serious pain to people in contact with the larvae (Rahmadsyah *et al*., 2006).

Natural pathogens play an important role in the regulation of *S. asigna* populations in oil palm plantations, since outbreaks of the insects are not infrequently terminated by epizootic disease in the pests. In Indonesia, epizootic outbreaks of *S. asigna* in oil palm plantations have been observed since 1996 (Rahmadsyah *et al*., 2003). Farmers collect the diseased larvae, store them in a freezer and, during outbreaks of *S. asigna*, spread them on oil palm leaflets. This has provided good control of the populations of pests in South Sumatra (Rahmadsyah *et al*., 2003), though the causal agent in *S. asigna* has not been identified.

In this study, we isolated a Thosea asigna virus (TaV) from *S. asigna* larvae suffering from epizootic disease in oil palm plantations in South Sumatra. Subsequently, we examined the pathogenicity of the purified TaV against Limacodidae larvae in Japan.

MATERIALS AND METHODS

Source of the agent causing epizootic disease

The *S. asigna* larvae suffering from epizootic disease were collected from oil palm plantations in South Sumatera, and were kept frozen at −80°C prior to processing for the experiments.

Diagnostic analysis using PCR with a primer set targeting a tetravirus

RNA was extracted from *S. asigna* larvae using Catrimox™ (Takara) according to the manufacturer's instructions and used as a template for reverse transcription-PCR (RT-PCR) with a One-Step RT-PCR kit (Invitrogen). RT-PCR was performed as recommended by the manufacturer using a primer set targeting the RdRp gene of TaV (Gen Bank accession No. AF 282930), G1 (5'-gagcaattag gagagtatcccg-3', nucleotides (nts) 343-364) and G2 (5'-tcg ggttgtttgcatctttagcctct-3', nts 572-597) (Fig. 1). The amplified DNA was then analyzed by agarose gel electrophoresis. The DNA fragments visualized with ethidium bromide staining were extracted from the gel, cloned into a pGEM-T Easy plasmid vector (Promega) and introduced into the competent cells of the *E. coli* strain DH5α as reported (Yamagishi *et al*., 1999). Plasmid DNAs prepared from the transformed bacterial colonies (white) in the agar plates containing X-Gal were sequenced using an automatic DNA sequencer (ABI PRISM 3130, Applied Biosystems).

Purification of viruses from the frozen *Setothosea asigna*

The purification of viral particles was carried out according to the method described by Yi *et al*., (2005) with minor modifications. In brief, the frozen insect larvae (200 g) were thawed and homogenized in 500 ml of extraction buffer (50 mM Tris/HCl, 1 mM EDTA (pH 7.5), and 0.2% 2-mercaptoethanol). The homogenate was clarified by centrifugation at 10,000 × g for 30 min, and the supernatant was then centrifuged at 100,000 × g for 3 h. The pellets were resuspended in 50 ml of TE buffer (50 mM Tris/HCl, and 1 mM EDTA (pH 7.5)), layered on TE buffer containing 30% sucrose (w/v), and then centrifuged at 100,000 × g for 3 h. The pellets were resuspended in 5 ml of TE buffer and subjected to further purification by a sucrose gradient (10 to 40% w/v, in TE buffer) spun at 100,000 × g for 2 h. The white band observed in the gradient was collected, centrifuged down at 100,000 × g for 3 h and then resuspended in distilled water.

The tentative viral fractions were observed to verify their purity and integrity by negative staining using a transmission electron microscope. In brief, viral particles purified by sucrose gradient centrifugation were negatively stained with 2% (w/v) sodium phosphotungstate (pH 7.2) on formvar-coated grids, and examined with a transmission electron microscope (Hitachi H-800).

Extraction of RNA from the purified particles, cDNA cloning and nucleotide sequencing

RNA was extracted from the purified viral particles using TRIzol (Invitrogen) reagent according to the manufacturer's instructions, except for the addition of glycogen to enhance RNA precipitation. The RNA pellet was resuspended in 10 μl of DEPC-treated water. The integrity of the RNA extracted from the particles was examined by electrophoresis on a 1% agarose gel containing formaldehyde (final concentration, 2.2 M). Complementary DNA (cDNA) against the RNA was then generated with random primers using a cDNA synthesis kit (Takara) according to the manufacturer's instructions. The resulting cDNAs were used as templates for the subsequent PCR. Two primer sets were used in the PCR. One set, 5'PRdRp (5'-cttcgtttagagggggtg-3', nts 1-19) and 3'PRdRp (5'-cgt ccccgcatgttag-3', nts 3752-3768), targeted the complete coding sequence for the RNA-dependent RNA polymerase gene (*RdRp*) of TaV (Gen Bank accession No. AF282930). The other set, CP1 (5' cgaatgatagtgcttttg 3', nts 85-102), and CP2 (5'-atcattttggaaaaaggtgcgc-3', nts 2461-2482), targeted the complete coding sequence for the capsid protein gene (*CP*) of TaV (AF062037). The PCR products were ligated into the pGEM-T Easy vector (Promega), and sequenced as above. Sequences of the inserted DNAs in the clones obtained in this study were analyzed using the computer programs BLAST (http://www.ncbi.nlm.nih.gov/) and Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin).

3' RACE analysis

The rapid amplification of cDNA ends (RACE) method was used to determine the complete nucleotide sequence of the 3' terminus of the RNA extracted from the particles. The RNA was polyadenylated with poly(A) polymerase (Invitrogen) in the presence of ATP before ligation with the RNA oligo adapter provided by the manufacturer. As a control, we carried out an experiment where polyadenylation at the 3'-terminus of the RNA was skipped. In the 3' RACE, the first strand of cDNA was synthesized using an oligo(dT) primer with a unique sequence at its 3'-terminus supplied by the manufacturer, and then amplified with a gene-specific primer, 3F (5'-ccagaggggtgc actgg-3', nts 2321-2340, AF062037) and GeneRacer™ 3' Primer (5'-gctgtcaacgatacgctacgtaacg-3') targeting the unique sequence of the oligo(dT) primer.

The PCR products were ligated into a TA TOPO cloning plasmid vector (Invitrogen), and the ligation mixture was used to transform One Shot® TOP10 Competent Cells

(Invitrogen). Plasmid DNA prepared from bacterial colonies was sequenced using the ABI PRISM 3130 (Applied Biosystem).

Pathogenicity of the purified virus against Limacodidae in Japan

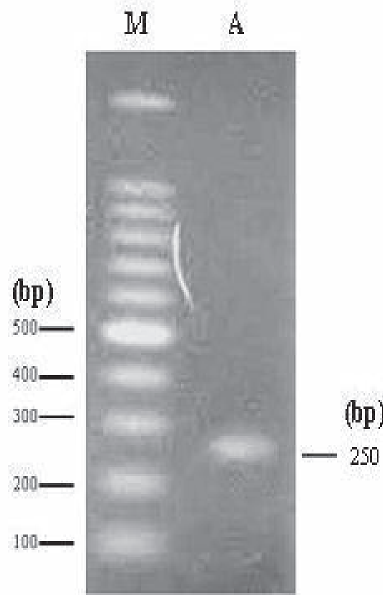
Limacodidae larvae of *M. flavescens* and *A. dentata* were collected from Maple, Cherry and Plum trees in Hokkaido University. Six larvae of each Limacodidae (various larval stages) were fed on the plum tree leaves (1 cm × 1 cm) treated with water, the water suspension of purified particles (1% v/v) and the water suspension of the diseased *S. asigna* , a homogenate of the moribund *S. asigna* larvae (10% w/w).

RESULTS

Identification and purification of TaV from carcasses of *S. asigna* larvae

In a preliminary experiment, RT-PCR followed by agarose gel electrophoresis yielded only one PCR product (Fig. 1 A). The DNA fragment was estimated to be about

(A)



(B)

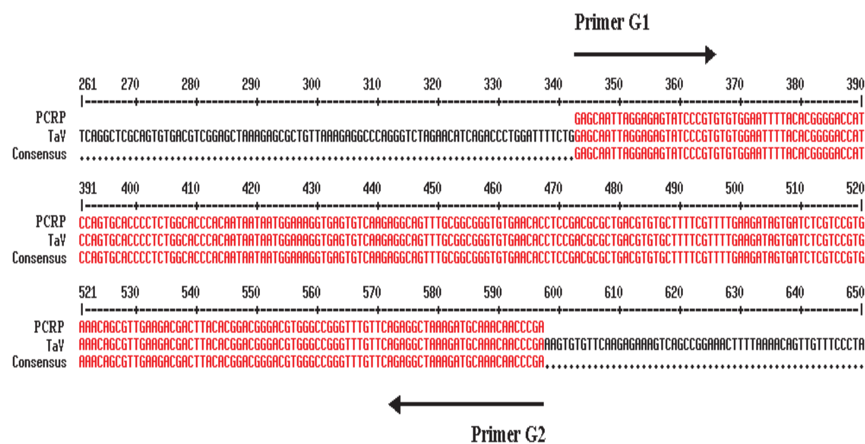


Fig. 1. RT-PCR using a primer set targeting the *RdRp* of TaV. **(A)** Agarose gel electrophoresis of PCR products (PCRP). Lanes M and A contain the markers and PCR products molecular, respectively. **(B)** The sequence of the PCR product of about 250 nts is aligned with the corresponding sequence of TaV. The positions of primers G1 and G2 are presented.

250 nts long, corresponding to the size of the target sequence in the TaV genomic RNA. A subsequent analysis revealed that the insert was 250 nts long and had a sequence that coincided with the target sequence of TaV (Fig. 1 B). The sequences obtained from three independent clones were the same.

Negative staining revealed that the purified particles had a spherical shape and no envelope, and were about 40 nm in diameter which was estimated from the width of tobacco mosaic virus (TMV) used as an internal size indicator (Fig. 2). These structural traits were similar to those reported for insect picorna-like viruses and members of the family Tetraviridae such as the *Nudaurelia capensis* β virus, TaV and *Dendrolimus punctatus* tetravirus (Bailey, 1976; Gordon *et al*., 1999; Hanzlik and Gordon, 1997; Hendry *et al*., 1968, 1995; Morris *et al*., 1979; Pringle *et al*., 1999; Tinsley and Melnick, 1973; Yi *et al*., 2005).

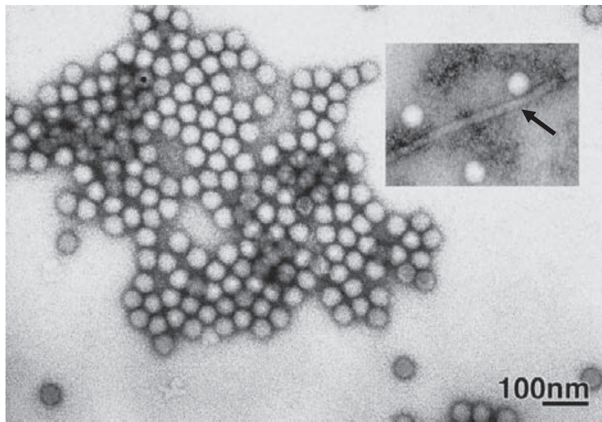


Fig. 2. Negative staining of particles purified from *S. asigna* by sucrose density gradient centrifugation. The insert shows the rod-shaped tobacco mosaic virus (TMV) used as an internal size indicator (⟵).

Sequencing analysis

Using the RNA extracted from the purified virus-like particles as a template, RT-PCR was carried out with the primer sets to amplify the RNA-dependent RNA-coding sequence (*RdRp*) and the capsid protein-coding sequence (*CP*) of TaV , resulting in the amplification of DNA fragments of expected sizes, 3858 nts (tentative *RdRp*) and 2482 nts (tentative *CP*) (data not shown). Subsequent sequencing revealed that the nucleotide sequence of the tentative *RdRp* and *CP* was about 99% identical to that of *RdRp* (ORF: 3883 nts) and *CP* (ORF: 2270 nts) of TaV, respectively (Fig 3). In addition, the overlapping of 627 nts was observed between the two sequences as is the case in TaV (Hanzlik and Gordon, 1997). A comparison of the TaV sequences showed that there were three nucleotide substitutions in the *RdRp* ORF, two of which resulted in amino acid substitutions (Fig. 3). Four nucleotide substitutions were observed in the *CP* ORF and one in the 3' terminal non-coding region. One of the five in the *CP* ORF was found to cause an amino acid substitution (Fig. 3).

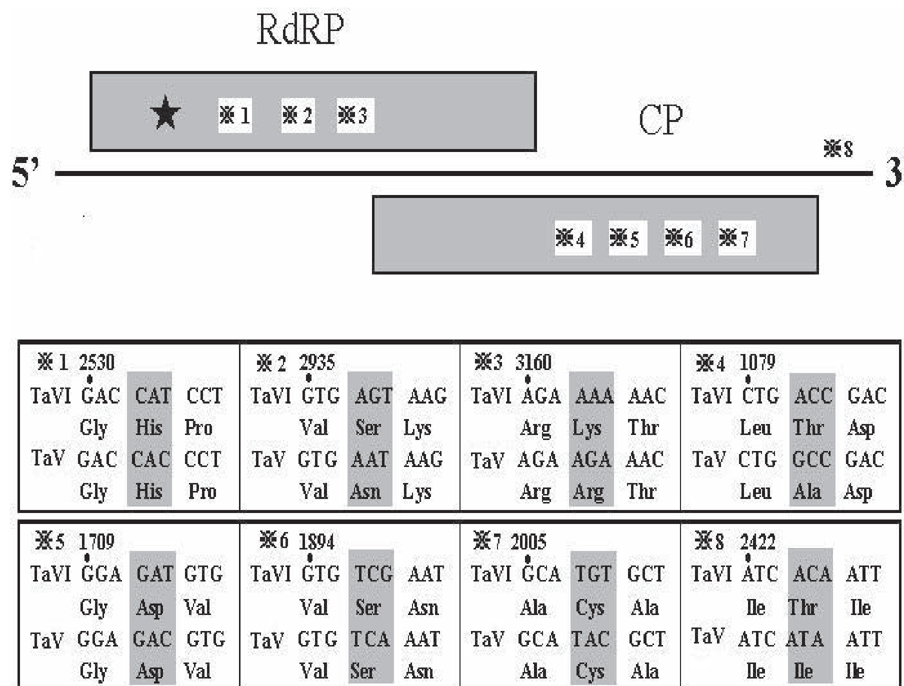


Fig. 3. A schematic representation of the genome's organization predicted from the sequence obtained in this study (TaVI). Boxes show the ORFs coding for RdRp and CP. The positions of a methyltransferase domain (★) and the nucleotide substitutions (※: synonymous and nonsynonymous) in comparison to the sequence of TaV (AF062037 and No. AF282930) (shadowed) are presented. The numbers (•) refer to the nucleotide number in the TaV.

Characteristics of the 3' terminus

To analyze the 3' terminal structure of the RNA extracted from the purified particles, the RACE method was adopted. When the RNA directly annealed with the RNA oligo adapters was used, no band was obtained in the subsequent PCR amplification for the 3' terminus (data not shown). However, polyadenylation at the 3'-terminus allowed amplification of two bands in the subsequent PCR, one with about 270 nts and the other with around 200 nts (Fig. 4). The sequence of the DNA fragment (272 nts) coincided with the sequence expected from the TaV genome reported previously (Gorbalenya *et al*., 2002; Pringle *et al*., 1999). The sequence of 200 nts could not be specified. These results strongly suggested that the RNA molecule extracted from the purified particles had no poly(A) stretch at the 3' terminus.

We also carried out 5' RACE (Fujita *et al*., 2006) to analyze the 5' terminal structure but obtained unreliable results (data not shown).

Pathogenicity of the isolate against Limacodidae larvae in Japan

The inoculation experiment showed that the isolate has the ability to infect Lomacodidae larvae in Japan, *M. flavescens* and *A. dentata*. *M. flavescens* larvae and *A. dentata* larvae inoculated with the crude homogenate of infected *S. asigna* larvae developed symptoms on the 1st and 3rd day post-inoculation, respectively. The same symptoms were observed in the larvae of both species on the 1st and 8th days after inoculation with the purified particles, respectively (Fig. 5).

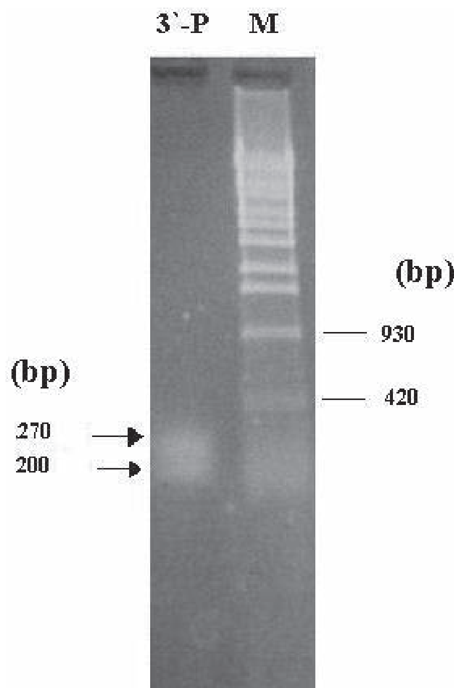


Fig. 4. Agarose gel electrophoresis of the PCR products in the 3' RACE analysis. Lane 3'-P contains the PCR products and M contains molecular markers. Arrowheads indicate the PCR-amplified fragments of about 270 nts and 200 nts.

The symptoms in the inoculated larvae were similar to those observed in the epizootic *S. asigna* larvae such as brown coloring, flaccidity and finally moribundity. In *M. flavescens*, the inoculation of the purified virions led to a moribund state without flaccidity, but that of the crude homogenate resulted in flaccid symptoms prior to moribundity (Fig 5). Flaccidity was observed for *A. dentata* even on infection with the purified virions.

DISCUSSION

Farmers in Indonesia use moribund *S. asigna* larvae collected in epizootic oil palm fields as a pesticide to control *S. asigna*. The method of application is very simple. About 100 grams of moribund larvae was homogenized, mixed with 12 liters of water and then sprayed on palm trees in the field. This method is cheaper than using commercially available pesticides. However, the epizootic agents in diseased larvae have not been identified. An aim of this study was to identify the causal agent of *S. asigna* infections in South Sumatera, Indonesia, in order to develop a biological pesticide for integrated pest management (IPM) in oil palm plantations.

Preliminary experiments demonstrated that the causal agent was filterable (data not shown), implying that it was a virus. We suspected that the epizootic infection was caused by TaV, the only virus reported for *S. asigna* in Indonesia (Pringle *et al*., 1999). Subsequent RT-PCR experiments using a primer set targeting a methyltransferase domain of the TaV RdRp gene resulted in the amplification of a DNA fragment with the size expected from the sequence of TaV. This result strongly supported the prediction about the causal agent. Using the procedure for purifying tetraviruses described by Yi *et al*, (2005), we obtained a fraction containing spherical virus-like particles around 40 nm in diameter which corresponded to TaV.

TaV is a RNA virus first isolated from *S. asigna* collected in an oil palm plantation in Malaysia (Reinganum *et al*., 1978). Further characterization including the nucleotide sequencing of TaV RNA was performed using TaV isolated in North Sumatera, Indonesia (Pringle *et al*., 1999). The nucleotide sequence for the cDNAs against the RNA extracted from the purified virus-like particles was almost the same as the nucleotide sequence reported for TaV (Gene Bank accession number: AF 282930) with the same organization consisting of two large open reading frames corresponding to the RdRp- and CP-coding regions of TaV which was overlapped by 627 nts (Fig. 3), indicating that the virus-like particles purified in this study were TaV.

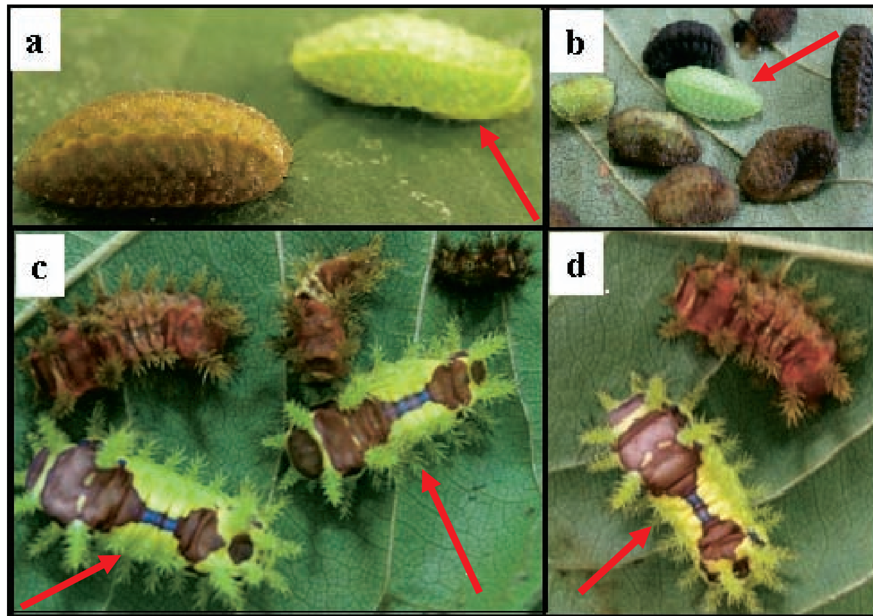


Fig. 5. Infection assay of TaV against Limacodidae larvae in Japan. *A. dentata* (a, b) and *M. flavescens* larvae (c, d), infected with purified TaV particles (a, c) and the water suspension of the diseased *S. asigna* (b, d). Green larvae (red arrows) were mock-infected (inoculated with water) as the control.

Eight nucleotide substitutions were observed in the ORFs, three in *RdRp* and five in *CP*. One of the three observed in *RdRp* was a synonymous substitution and the other two resulted in the replacement of asparagine (Asn) with serine (Ser) and arginine (Arg) with lysine (Lys) (Fig. 3). These substitutions occurred among amino acids with similar physicochemical properties, suggesting they caused no significant change in the structure of RdRp whose function was critical to the viral replication. In *CP*, only one of the five substitutions caused an amino acid change, from alanine (Ala) to threonine (Thr) (Fig. 3), which resulted in a change from a hydrophobic to hydrophilic residue. Though this substitution may have a functional effect on CP, there is not enough information to predict the possible functional change at present.

The 3' RACE was successful only when a poly(A) tail was added at the 3' terminus, suggesting that there was no poly(A) tract at the 3' terminus of the viral RNA, consistent with a previous report for TaV (Pringle *et al*., 1999). On the other hand, the 3' terminal sequences of the genomic RNAs of tetraviruses for which complete nucleotide sequences are available can be folded into 3'-tRNA-like secondary structures (Gordon *et al*., 1995). The poly(A) tail at the 3' terminus protects the RNA from degradation by exonucleases (Gordon and Hanzlik., 1998), and is involved in the initiation of translation (Beelman and Parker, 1995; Dreytus and Philippe, 2002). Considering these observations, the lack of a poly(A) tail in TaV RNA, like the hepatitis A virus (HAV) (Kusov *et al*., 2005), implied a unique mechanism of replication/multiplication among tetraviruses. The 5' end of TaV remains to be characterized (Gorbalenya *et al*., 2002).

TaV and other members of the family Tetraviridae are characterized by the ability to infect midgut cells (Gordon and Hanzlik, 1998), probably related to the prompting of infected *S. asigna* larvae such as feeding—termination and liquefying. TaV shows greater infectivity in the early stages of larval development than the later stages (Entwistle, 1987; Rahmadsyah *et al*., 2003). These traits suggest that TaV has a good potential as a viral insecticide in Integrated Pest Management (IPM) (Hendry and Agrawal, 1993; Christians *et al*., 200l). Interestingly, TaV showed strong pathogenicity against Limacodidae larvae in Japan, *M. flavescens* and *A. dentata*, suggesting it to be a novel biological control agent for the Limacodidae pest which feeds on economically important plants in Japan such as cherry, plum and maple trees. This is the first report of the ability of TaV to infect insect species other than *S. asigna*. These observations also suggested that TaV has an excellent potential as a control agent against Limacodidae insects.

Deep insight into the molecular mechanism of their replication should facilitate the effective/safe application of viruses. The Tetraviridae include the *Betatetravirus* and *Omegatetravirus*, the former has a monopartite RNA genome and the latter a bipartite genome, suggesting a difference in the replication mechanisms between them (Agrawal and Johnson, 1992; Hanzlik *et al*., 1993, 1995, 2005). A recent study showed that the *Euprosterna elaeasa* virus (EeV) is closely related to TaV and the two share properties distinguishing them from prototypic tetraviruses, including a predicted conserved pseudonot at the

3’-end, a structurally unique RdRp with a permuted active site (pRdRp) and a putative VPg signal mediating the priming of RNA synthesis in dsRNA Birnaviridae (Zeddam *et al*., 2010). These observations suggested unique replication mechanisms of TaV and EeV among tetraviruses. However, the replication mechanisms of TaV/EeV remain to be delineated as well as other members of Tetraviridae, mainly becausethere is a lack of susceptible cell lines with the exception of the Providence virus (PrV) (Pringle *et al*., 2003).

In conclusion, the virus isolated from the epizootic *S. asigna* larvae collected in South Sumatra was a TaV, which also showed strong pathogenicity against Limacodidae larvae in Japan. These results will greatly facilitate the study of TaV as a promising biological agent to control Limacodidae pests in oil palms and other plants.

ACKNOWLEDGEMENTS

We thank Mr. Y. Yamada (Field Science Centre for Northern Biosphere, Hokkaido University) for collecting Limacodidae larvae in the field. This work was supported in part by Bilateral Research Program from the Japan Society for the Promotion of the Science.

REFERENCES

Agrawal, D.K. and Johnson, J.E. (1992) Sequence and analysis of the capsid protein of *Nudaurelia capensis* ω virus, an insect virus with $T=4$ icosahedral symmetry. *Virology*, **190**, 806-814.

Bailey, L. (1976) Viruses attacking the honey bee. *Adv. Virus. Res*., **20**, 271-304.

Beelman, C.A. and Parker, R. (1995) Degradation of mRNAin eukaryotes. *Cell*, **81**, 179-183.

Christians, P.D., Dorrian, S.J., Gordon, K.H.J. and Hanzlik, T.N. (2001) Pathology and properties of the tetravirus *Helicoverpa armigera stunt virus. Biol. Control*.., **20**, 65-75.

Dreytus, M. and Philippe, R. (2002) The poly(A) tail of mRNAs ; bodyguard in eukaryotes, scavenger in bacteria. *Cell*, **111**, 611-613.

Entwistle, P.F. (1987). Virus diseases of Limacodidae. In *Slug and Nettle Caterpillar*. (Cock, M.J.W., Godfray, H.C.J., and Holloway, J.D, eds.), pp. 213-221, C.A.B. International, Wallingford.

Fujita, R., Matsuyama, T., Yamagishi, J., Sahara, K., Asano S. and Bando, H. (2006) Expression of *Autographa californica multiple nucleopolyhedrovirus* genes in mammalian cells and upregulation of the host beta-actin gene. *Virology*, **80**, 2390-2395.

Godfray, H.C.J., Cock, M.J.W. and Holloway, J.D. (1987) An introduction to the Limacodidae and their bionomics. In *Slug and Nettle Caterpillar* (Cock, M.J.W., Godfray, H.C.J., and Holloway, J.D, eds.) pp. 1-8, C.A.B. International, Wallingford,

Gorbalenya, A.E., Pringle, F.M., Zeddam, J.L., Luke, B.T., Cameron, C.E., Kalmakoff, J., Hanzlik, T.N., Gordon, K.H. and Ward, V.K. (2002) The palm subdomain-based active site in internally permuted in viral RNA-dependent RNA polymerase of an ancient lineage. *J. Mol. Biol*., **324**, 47-62.

Gordon, K.H.J. and Hanzlik, T.N. (1998) Tetravirus. In *The Insect Viruses* (Miller, L.K. and Ball, L.A. eds.), pp. 269-299, Plenum Press, New York and London.

Gordon, K.H.J., Johnson, K.N. and Hanzlik, T.N. (1995) The larger genomic RNA of Helicoverpa armigera stunt tetravirus encodes the viral RNA polymerase and has a novel 3’-terminal tRNA-like structure. *Virology*, **208**, 84-98.

Gordon, K.H.J., Williams, M.R., Hendry, D.A. and Hanzlik, T.N. (1999) Sequence of the genomic RNA of *Nudaurelia capensis* β virus (Tetraviridae) defines a novel virus genome organization. *Virology*, **258**, 42-53.

Hanzlik, T.N., Gordon, K.H.J., Gorbalenya, A.E., Hendry, D.A., Pringle, F.M., Ward, V.K. and Zeddam, J.L. (2005) Family tetraviridae. In *Virus Taxonomy, Eighth report of the International Committee on Taxonomy of Viruses* (Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A. Eds.), pp. 877-883, Elsevier, Academic Press, Amsterdam,

Hanzlik, T.N., Dorrians, S.J., Gordon, K.H.J. and Christian, P.D. (1993) A novel small RNA virus isolated from the cotton bollworm, *Helicoverpa armigera*. *J. Gen. Virol*., **74**, 1805-1810.

Hanzlik, T.N., Dorrians, S.J., Johson, K.N., Brooks, E.M. and Gordon K.H.J. (1995) Sequence of RNA2 of the *Helicoverpa armigera* stunt virus (Tetraviridae) and bacterial expressions of its genes. *J. Gen. Virol*., **76**, 799-811.

Hanzlik, T.N. and Gordon, K.H.J. (1997) The Tetraviridae. *Adv. Virus. Res*., **48**, 101-168.

Hendry, D.A. and Agrawal, D.K.. (1993) Tetravirus. In *Encyclopedia of Virology*. pp. 1416-1422, Academic Press. New York.

Hendry, D.A., Becker, M.F. and Vam Regenmortel, M.H.V. (1968) A non-inclusion virus of the pine emperor moth *Nudaurelia cytherea capensis* Stoll. *S. Afr. Med. J*., **42**, 117-117

Hendry, D.A., Johnson, J.E., Rueckert, R.R., Scotti, P.D. and Hanzlik, T.N. (1995) Family tetraviridae. In *Virus Taxonomy: six Report of the International Committee on Taxonomy of Viruses* (Murphy, F.A., Fauquet, C.M., Bishop, D.L., Ghabrial, S.A. , Jarvis, A.W., Martelli, G.P., Mayo, M.A. and Summers, M.D. eds.), pp. 372-375, Springer-verlag, New York.

Holloway, J.D., Cock, M.J.W. and Desmier de Chenon, R. (1987). Systematic account of South-east Asian pest Limacodidae. In *Slug and Nettle Caterpillar* (Cock, M.J.W., Godfray, H.C.J., and Holloway, J.D, eds.) pp. 64-66, C.A.B. International. Wallingford.

Kusov, Y.Y., Rainer, G. and Verena, G.M. (2005) Replication and in vivo repair of the hepatitis A virus genome lacking the poly (A) tail. *J. Gen. Virol*., **86**, 1363-1368.

Morris, T.J., Hess, R.T. and Pinnock, D.E. (1979) Physicochemical characterization a small RNA virus associated with baculovirus infection in *Trichoplusia ni*. *Intervirology*, **11**, 238-247.

Pringle, M.F., Gordon, K.H.J., Hanzlik, T.N., Kalmakoff, J., Scotii, P.D. and Ward, V.K. (1999) A novel capsid expression strategy for Thosea asigna virus (Tetraviridae). *J. Gen. Virol*., **80**, 1855-1863.

Pringle, M.F., Johnson, K.N., Goodman, C.L., Mcintosh, A.H. and Ball, L.A. (2003) Providence virus : a new member of the Tetraviridae that infects cultured insect cells. *Virology*, **306**, 359-370.

Rahmadsyah., Agustinus, T.W.P., Nurbianto., Maria, S. and Ilham, H.K. (2003) Integrated pest management of leaf lest in Tania Selatan Plantation. Indonesian Entomology Association Congress VI and Entomology Symposium: *Sustainability of Insect Management*. Cipayung, Indonesia (In Indonesian)

Rahmadsyah., Agustinus, T.W.P. and Maria, S. (2006). *Population of leaf pest in Tania Selatan Plantation*. (unpublished annual report). Tania Selatan Plantation. Indonesia (In Indonesian)

Reinganum, C., Robertson, J.S. and Tinsley, T.W. (1978) A new group of RNA Virus from insects. *J. Gen. Virol*., **40**, 195-202.

Sipayung, A. (1987). Pest Management of oil palm plantation in Indonesia. *Proc Seminar of Management in Oil Palm Industry*. Medan, 173-183 (In Indonesian)

Tinsley, T.W. and Melnick, J.L. (1973) Potential ecological hazards of pesticidal viruses. *Intervirology*, **2**, 206-208.

Yamagishi, J., Hu, Y., Zheng, J. and Bando, H. (1999) Genome organization and mRNA structure of *Periplaneta fuliginosa* densovirus imply alternative splicing involment in viral gene expression. *Arch. Virol*., **144**, 2111-2124

Yi, F.M., Zhang, J.M., Yu, H.Y., Liu, C.F., Wang, J.P. and Hu, Y.Y. (2005) Isolation and identification of a new tetravirus from *Dendrolimus punctatus* larvae collected from Yunnan Province, China. *J. Gen. Virol*., **86**, 789-796.

Zeddam, J.L., Gordon, K.H.J., Lauber, C., Alves, A.F., Luke, B.T., Hanzlik, T.N., Ward, V.K. and Gorbalenya, A.E. (2010) *Euprosterna elaeasa* virus genome sequence and evolution of the Tetraviridae family: Emergence of bipartite genomes and conservation of the VPg signal with the dsRNA Birnaviridae family. *Virology*, **397**, 145-154.