

Occurrence of reovirus in oak tasar silkworm *Antheraea proylei*

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Infection of cytoplasmic polyhedrosis virus (CPV, Reoviridae) in the oak tasar silkworm *Antheraea proylei* (Saturniidae) has been recorded. CPV forms hexahedral shaped polyhedra with a mean diameter of $10.75 \pm 1.2 \mu\text{m}$. The viral genome is fragmented and has eleven segments of double-stranded RNA in equimolar concentrations. The size of the dsRNA fragments ranged from 4.0 to 0.56 kbp; the total genome size is 24.21 kbp. This is the first report on the occurrence of reovirus in the wild silk moth *A. proylei*.

ANTHERAEA PROYLEI, commonly known as oak tasar silkworm is a domesticated species in Manipur (Northeast India). *A. proylei* is a viable hybrid between Indian wild silk moth *A. assama* (♀) and its Chinese counterpart *A. pernei* (♂). A number of pathogens infect the larvae during development and, as a consequence, the silk yield is affected. Among them, baculovirus are important and they infect *A. proylei* more frequently than other viral pathogens (Regional Tasar Research Institute, RTRI)¹. In general, the viral disease of insects are due to the viral pathogens belonging to the following families, viz. Ascoviridae, Baculoviridae, Poxviridae, Iridoviridae, Polydnviridae, Nodaviridae, Tetraviridae, Picornaviridae and Reoviridae; of these, the first five families have DNA as genome and the rest are RNA virus^{2,3}. The cytoplasmic polyhedrosis virus of *A. proylei* (ApCPV) belongs to Reoviridae. In this paper the occurrence of CPV in the oak tasar silkworm is reported for the first time. Further, structure of the polyhedral inclusion bodies (PIBs) of ApCPV and nature of the viral genome are also presented.

A. proylei infected by virus were obtained from RTRI, Imphal (750 mmsl), Manipur. The PIBs from the infected larvae were suspended in water at the concentration of $5 \times 10^5/\text{ml}$. Fresh tender leaves of the oak plant (*Quercus serrata* Thung.) were dipped in a suspension containing PIBs and later the leaves were air dried. PIBs-coated leaves were fed to III instar larvae of *A. proylei* and the larvae were reared under laboratory conditions of $23 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ RH.

Viral infected dead larvae (about twenty) were collected and allowed to putrefy. The cadavers were filtered

through cheese cloth after two weeks of putrefaction and subjected to centrifugation at 600 rpm for a few minutes in a swingout rotor. The supernatant was collected and centrifuged at 10,000 rpm for 5 min to pellet PIBs. The pelleted PIBs were subjected to further purification following the standard protocols⁴. Finally, the purified PIBs were suspended in dd H₂O and stored at -20°C till use. To extract the viral genome, the polyhedra ($10 \times 10^9/\text{ml}$) were incubated for one hour at 56°C with 1% SDS-phenol. The aqueous phase was extracted with phenol-chloroform and the genome was precipitated with 2.5 volumes of 100% ethanol. The pellet was washed with 70% ethanol, dried and suspended in TE (pH 7.5)⁵.

The genome samples were resolved in 1.2% agarose gel with $1 \times \text{TAE}$ buffer at 40 V for 10 h and the gel was stained with ethidium bromide⁶ and photographed. The viral genome was also resolved in 8% continuous polyacrylamide gel⁶ with $1 \times \text{TAE}$ buffer at 50 V for 48 h and the gel was stained with silver nitrate⁷.

The PIBs were dehydrated in an ethanol series, air dried on a glass slide and processed for scanning electron microscopy as described by Adams and Wilcox⁸. The processed PIBs were scanned in Joel scanning electron microscope and the pictures were documented.

To understand the nature of the viral genome, it was subjected to the following treatments. The viral genome was aliquoted into seven tubes each containing 1.5 μg . Of these aliquots, two were incubated with 0.1 N and 0.2 N (final concentration) KOH for 5 min; another two more aliquots were incubated with 10 μg and 20 $\mu\text{g}/\text{ml}$ of RNase A in the presence of $2 \times \text{SSC}$ (high ionic strength) at 37°C for 2 h. Another aliquot was incubated with 10 $\mu\text{g}/\text{ml}$ of RNase A without SSC (low ionic strength) at 37°C for 3 h. (dsRNA is sensitive to RNase A under low ionic strength and it is resistant under high ionic strength^{9,10}.) Two other aliquots were incubated with 400 ng and 800 ng/ml of DNase I at 37°C for 2 h. All the samples, after the stipulated incubation periods, were electrophoresed in 1.2% agarose gel. The viral genome was also subjected to thermal denaturation study following Mandel and Marmur¹¹ protocol in the presence of $0.1 \times \text{SSC}$.

The EM analysis of the PIBs indicated that the size of polyhedra ranged from $8.34 \mu\text{m}$ to $12.24 \mu\text{m}$, with a mean of $10.75 \mu\text{m}$ and their shape is hexahedral (Figure 1).

The viral genome was characterized based on the results of the following experiments: (i) Viral genome was resolved in agarose gel (Figure 2). Lanes 1 and 2 show the pattern of separation obtained by loading 1.5 μg of viral genome. The genome was not digested with any restriction endonuclease before electrophoresis; still the genome showed clear separation of fragments of size ranging from 4.0 to 0.56 kbp. The pattern of

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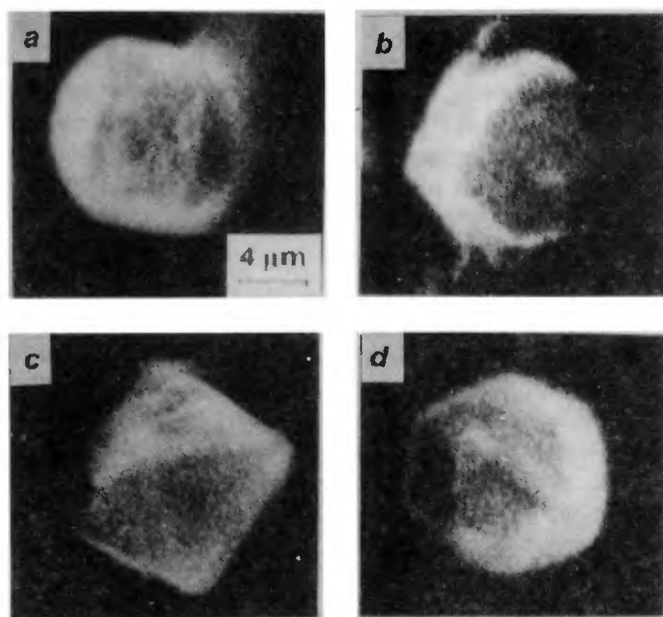


Figure 1. Scanning electron microscopic (SEM) pictures showing different angles of the hexahedral shape PIBs of ApCPV (1800 \times).

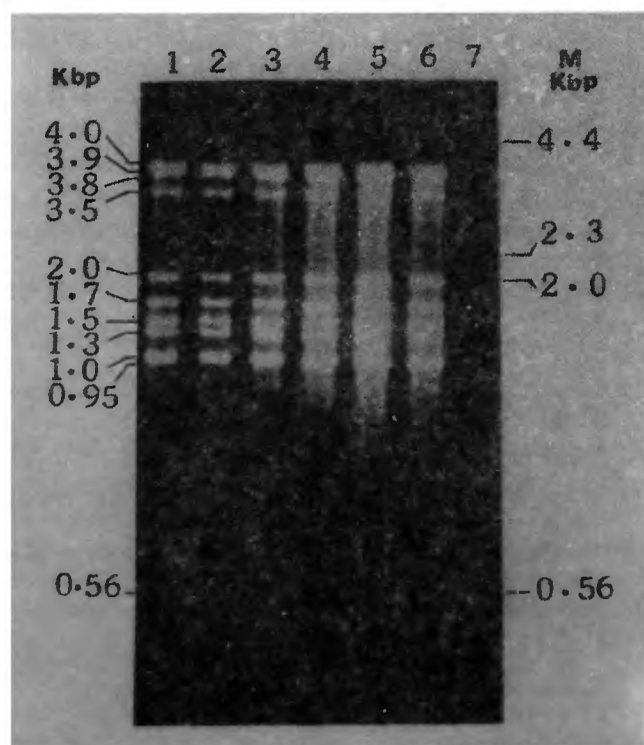


Figure 2. Electropherogram of ApCPV genome; The ApCPV genome subjected to different experimental conditions was resolved in 1.2% agarose gel at 40 V for 10 h. About 1.5 μg of genome was loaded in each lane. Lanes 1 & 2: ApCPV genome (undigested); Lanes 3 & 4: ApCPV genome digested with 400 ng and 800 ng/ml of DNase I; Lanes 5 & 6: ApCPV genome digested with 10 μg, 20 μg/ml of RNase A at high salt concentration. Lane 7: ApCPV genome digested 10 μg/ml of RNase A at low salt concentration. M, Schematic representation of λDNA digested with *Hind*III.

separation confirms that the viral genome is fragmented in nature and the first three fragments and the ninth and tenth fragments are co-migrating. To resolve clearly the co-migrating fragments, the genome was separated in 8% polyacrylamide gel and stained with silver nitrate. The co-migrative region contained 4.0, 3.9 and 3.8 kbp fragments in the higher segment and 1.0 and 0.95 kbp fragments in the lower segment. The genome has eleven fragments and the total size averaged to 24.21 kbp. This picture confirms the equimolar concentration of each fragment (Figure 3). (ii) On incubating the genome with KOH, it was found that the genome was sensitive to alkali (figure not shown). (iii) DNase I digestion of the genome showed that it is resistant to DNase I (Figure 2; lanes 3 and 4). These two observations of alkali and DNase I treatments confirmed that the viral genome is RNA. (iv) Aliquots of viral genome subjected to RNase

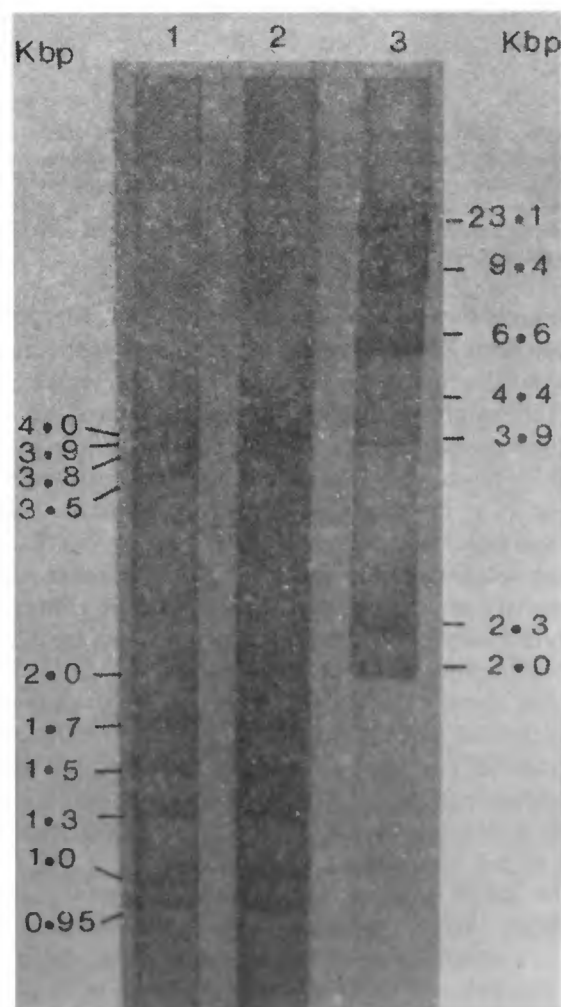


Figure 3. The ApCPV genome was resolved in 8% polyacrylamide gel at 50 V for 48 h and stained with AgNO₃. Lane 1: ApCPV genome 0.5 μg/lane; Lane 2: ApCPV genome 1 μg/lane; Lane 3: λDNA digested with *Hind*III and pBR 322 plasmid digested with *Hind*III (marker).

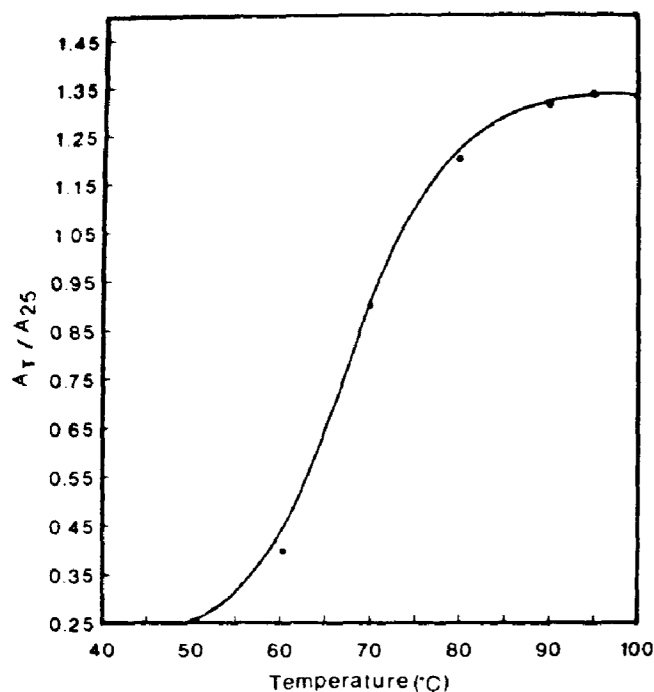


Figure 4. Thermal denaturation studies of the viral genome: The viral genome shows hyperchromicity at 260 nm wavelength with the increase of temperature. A_{25} , initial absorbancies of the genome sample at 25°C. A_T , the absorbancies of genome sample at the 50, 60, 70, 80, 90, 95 and 100°C. A_T/A_{25} means the absorbancies of genome at respective temperature divided by the absorbance at 25°C.

A treatment was resolved in the agarose gel. The genome treated with RNase A under high ionic strength showed resistance to the endoribonuclease activity (lanes 5 and 6). The genome subjected to RNase A under low ionic strength resulted in complete degradation of the genome (lane 7). It is already known that at high salt concentration RNase A is inactive against dsRNA, while at low salt concentration, it is active against dsRNA. (v) Thermal denaturation studies of the genome revealed hyperchromicity at 260 nm with the increase of temperature (Figure 4). These observations confirmed that the genome is double stranded RNA.

From these experimental data, the viral genome is characterized as follows; (i) the genome is dsRNA and fragmented into eleven segments; (ii) the size of the fragments ranged from 4.0 to 0.56 kbp; (iii) the fragments are in equimolar concentration and (iv) the total molecular size of the viral genome is 24.21 kbp.

The nature of the genome (dsRNA and segmented) confirms that the virus belongs to Reoviridae family and is a cytoplasmic polyhedrosis virus (CPV). Generally, the polyhedra of CPVs are heterogeneous in shape and size⁵. For instance, the CPVs isolated from the infected larvae of *Euxoa scandens*¹² and *Trichoplusia ni*¹³ showed presence of spherical polyhedra, whereas their respective cells in culture produced cuboidal-shaped polyhedra. On

the contrary, the shape of *Helicoverpa armigera* CPV PIBs was hexahedral⁵. Thus, the PIBs of CPVs exhibit differences in shape which could be due to virus and also the host cells⁵. The present observation of segmented genome of ApCPV is further corroborated by the occurrence of segmented genome as in other CPVs, e.g. *B. mori* CPV has nine segments. *E. scandens* CPV and *H. armigera* CPVs have 10 segments each. The CPV-infected *A. proylei* larvae showed a restless behaviour and they died after 10 or 12 days of the virus acquisition. The virulence of CPV has been found to be low but on co-infection with bacterial or other insect viral pathogens, its virulence has been reported to be very high¹⁴. In addition, the CPV infection can also be transmitted from one generation to another^{15,16}. Looking at the above points, the viral disease is significant and deserves much attention.

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