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Characterization of a Baculovirus Newly Isolated from the Tea Slug Moth, *Iragoidae fasciata*

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The tea slug moth *Iragoidae fasciata* (Lepidoptera, Eucleidae) is one of the main insect pests that attack tea bushes. A new nucleopolyhedrovirus (NPV) called *Iragoidae fasciata* NPV (IrfaNPV) was recently isolated from diseased larvae. An 11,626 bp fragment of the viral genomic DNA containing the *polyhedrin* gene and other 12 genes was cloned and sequenced. Gene comparison and phylogenetic analysis showed that IrfaNPV is a member of the Group I NPVs. However, the genomic organization of IrfaNPV is highly distinct. In addition, electron microscopy analysis showed that IrfaNPV is a single nucleocapsid NPV (SNPV). An inoculation assay showed that IrfaNPV is semi-permissive in the *Trichoplusia ni* cell line Tn-5B1-4. Bioassays on lethal concentration (LC_{50}) and lethal time (LT_{50}) were conducted to test the susceptibility of *I. fasciata* larvae to the virus.

***Keywords*:** *Iragoidae fasciata*, nucleopolyhedrovirus, SNPV, *polyhedrin* region, phylogeny, permissive cell line

Baculoviridae is a diverse family of pathogens that infect arthropods, particularly insects of the order Lepidoptera. Two genera, nucleopolyhedrovirus (NPV), and granulovirus (GV), have been recognized and are distinguished from one another by the morphology of their occlusion bodies. NPVs are designated single (S) or multiple (M) based on the number of nucleocapsids contained within their virions (Blissard *et al*., 2000). NPVs have been subdivided into groups I and II based on molecular phylogeny (Zanotto *et al.*, 1993). NPVs typically produce progeny viruses with two different phenotypes: occlusion-derived viruses (ODV) and budded viruses (BV). ODV transmits infection from insect to insect by infecting midgut columnar epithelial cells, whereas BV causes systemic infection within the host. The two viral forms are essential for natural propagation of baculoviruses.

The tea slug moth *Iragoidae fasciata* (Lepidoptera, Eucleidae) is one of the main insect pests that attack tea bushes in eastern Asia. This insect pest has caused a serious decrease in tea production during some years, and its larvae have stinging hairs capable of causing a rash in farm workers. A new NPV was recently isolated from diseased larvae of *Iragoidae fasciata.* It is well known that baculovirus infection is one of the factors that cause fluctuations in susceptible insect populations in the field. Baculoviruses have the potential to be used as biopesticides. For example, an NPV (EcobNPV) isolated from the tea looper caterpillar *Ectropis oblique* has been successfully used in the biological control of the tea bush pest (Ma *et al*., 2006). IrfaNPV isolated from the tea slug is also being developed as a biopesticide for the control of this insect pest. To achieve a better understanding of this virus and to provide a basis for the development of the virus as a biopesticide or an engineered pesticide, the morphology, phylogeny and infectious properties of the virus were observed and analyzed in this study.

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Materials and Methods

Virus and DNA

The virus used in this experiment was isolated from diseased larvae of *I. fasciata* in Hangzhou, Zhejiang Province in the People's Republic of China. The virus was propagated in 4th-instar larvae of *I. fasciata* and occlusion bodies were purified by sucrose-gradient centrifugation (O'Reilly *et al.*, 1992). Viral genomic DNA was isolated from purified occlusion bodies by dissolution in 0.1 M Na_2CO_3 and 0.01 M NaCl (pH 10.5), followed by proteinase K and SDS treatment, phenol-chloroform extraction, and precipitation in ethyl alcohol. The DNA was then dissolved in 0.1× TE buffer.

Cell culture

The *Trichoplusia ni* cells Tn5B-1-4 (High Five) (Invitrogen, USA) were maintained at 27°C in TNM-FH insect medium (Sigma, USA) with 10% fetal bovine serum.

Electron microscopy

To examine the appearance of the polyhedra, the purified polyhedra were directly observed under a transmission electron microscope. To examine the shape of occlusion-derived virus (ODV), the purified polyhedra were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer, and post-fixed in 1% osmium tetroxide in the same buffer. Fixed samples

were dehydrated through a graded series of ethanol solutions and embedded in Spurr's resin. Sections were cut, stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (JEOL, Japan).

Cloning of the fragment containing the polyhedrin gene

Viral genomic DNA was doubly digested with the restriction endonucleases *Eco*RI, and *Xho*I (TaKaRa Co., Japan). The digested fragments were inserted into the pSK+ vector (Novagen, Germany) with T4 DNA ligase (Promega, USA) to build a genomic DNA library. The 45 clones were screened for *polyhedrin* separately by PCR with *Taq* polymerase (Promega, USA) and dNTPs (TaKaRa Co., Japan) using a pair of primers corresponding to the conserved region of the baculovirus *polyhedrin* gene, PolhMF; 5'-AARCCHGAC ACVATGAA-3' and PolhMR; 5'-GYTTRTARAAGTTYTCC CA-3'. A positive clone for *polyhedrin* was sequenced using the universal primers M13/pUC forward and M13/pUC reverse. The obtained sequence was compared with the sequences in the GenBank using the BLAST program.

Phylogenetic analysis

Baculovirus gene comparison was performed with sequences from GenBank and published papers. Phylogenetic trees for the *lef*2, *polyhedrin*, *pk*-1, and *ie*-1 genes were constructed by the N-J method (PAUP* 4.0, beta 10 version, Sinauer Associates, Inc., USA) with the default settings for random break tie and the distance option for mean character difference. Statistical support for each node was evaluated by bootstrap analysis with 1,000 replicates. The tree was refined by using TREEVIEW (V. 1.6.6, http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The nucleotide sequence reported here was submitted to GenBank under accession number FJ362523.

Infection of TN-5B1-4 cells with IrfaNPV

BV of the IrfaNPV was isolated from the hemolymph of virus-infected *I. fasciata* larvae. After 10 continuous passages, the supernatant of the culture was used for further infection tests. A monolayer of Tn-5B1-4 cells was seeded in a 24 well plate. Cells (10^5) were infected with 10 μl of the supernatant containing IrfaNPV BVs. Cells (10^5) were also infected with the control virus, *Autographa californica* NPV (AcMNPV) C6 clone at an MOI of 1. For the time course analysis of proliferation, the infected cells were harvested at the designated times and washed with 1× PBS (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) three times and then total DNA for each well was extracted using the Universal Genomic DNA Extraction kit ver. 3.0 (TaKaRa Co., Japan). The supernatant of each well was also collected and centrifuged at 90,000×g for 1 h. The pellet was used for virus DNA extraction using the same DNA extraction kit. Each treatment was performed in triplicate.

Real-time quantitative PCR

Two pairs of primers were designed based on the sequenced *if-ac38* gene region of IrfaNPV (GenBank accesion no. FJ362523) and the *conotoxin-like* (*ctl*) gene region of AcMNPV genomic sequence (GenBank accession no. NC_001623). The primer set for the detection of IrfaNPV were If-ac38-F; 5'-ATGAAAAACGCTGCAGCAGG-3', position 1530-1549 nt in GenBank FJ362523 and If-ac38-R; 5'-GGAATCGCAACAG TCTCGAT-3', position 1391-1410 nt in GenBank FJ362523, and the expected amplified fragment was 159 bp long. The primers for the detection of AcMNPV were Acctx-F; 5'-GGATCCATGCAAATCAAAACTTAC-3', position 2245-2227 nt in GenBank NC_001623 and Acctx-R; 5'-CTCGAGTTATT GTGGTAAGCAATAA-3', position 2102-2084 nt in GenBank NC_001623, the expected size of amplified fragment was 163 bp. Real-time quantitative PCR (qPCR) was conducted on an iCycler iQ instrument (BIO-RAD, USA) using the SYBR *Premix Ex Taq* kit (TaKaRa Co., Japan) according to the manufacturer's protocol. Each amplification reaction was performed using a 25 μl reaction mixture under the following conditions: denaturation at 95°C for 1 min, followed by 40 cycles of a 95°C for 10 sec and at 60°C for 20 sec. A heat-dissociation was performed on the PCR products (temperature range, 60~95°C) during the last step of each cycle. Following amplification, melting curves were constructed, and data analysis was performed by using the iCycler iQ optical system software package version 3.1 (Bio-Rad, USA). All genomic DNA samples, PBS-treated negative controls and no-templated controls (NTC) were carried out in triplicate, and the average threshold cycle (Ct) value was used to quantify the relative IrfaNPV and AcMNPV copy numbers.

Bioassays

The occlusion bodies were suspended in sterile water at a concentration of 1×10^7 PIB/ml. A series of 10-fold dilutions was prepared from the OB stock solution. Five concentrations (1×10^7 PIB/ml, 1×10^6 PIB/ml, 1×10^5 PIB/ml, 1×10^4 PIB/ml, 1×10^3 PIB/ml) were used for the bioassay. Twigged tea leaves were dipped in their respective concentrations of PIB suspensions, allowed to air dry at room temperature, and were then fed to larvae. The leaves treated with sterile water were used as a control. Bioassays were performed by continuous feeding of the OB to 3rd instar larvae of *I. fasciata* on fresh tea leaf surfaces. Larvae were fed on normal fresh diets at 3 days post-inoculation. These larvae were observed daily until they died or pupated. Experiments were performed with 60~75 larvae per dose in triplicate. All analyses, including evaluation of virulence indices (LC50 and LT50), were performed using DPS software (Feng *et al*., 1998).

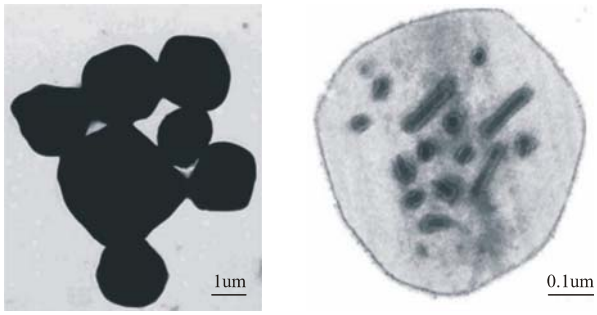

Fig. 1. Electron microscope observations of the IrfaNPV.

Table 1. ORFs in the *Eco*RI/*Xho*I 11,626 kb region of the IrfaNPV genome

Position	Gene	Strand and promoter	Amino acid length	AcMNPV homologous ORFs	Best matched Baculovirus ORFs		
					Baculovirus and gene name	Identical aa number/length (%)	Score (bist)
1-604	*39k*	−None	>203	36	MaviMNPV 39k	111/183 (60%)	221
598-939	*lef 11*	−L	113	37	AcMNPV lef11	65/113 (57%)	124
899-1543	*if-ac38*	−L E	212	38	PlxyMNPV orf38	156/206 (75%)	326
1576-2094	*ptp*	+E	172	1	BmNPV ptp	95/168 (56%)	228
2129-2413	*if-ac5*	−L	94	5	AcMNPV orf5	28/88 (31%)	50.8
2382-3029	*lef2*	+L	237	6	BmNPV lef2	113/214 (52%)	223
3125-3868	*polyhedrin*	+L	247	8	AnpeNPV polyhedrin	235/247 (95%)	489
3871-5571	*vp78*	−L	566	9	AcMNPV orf1629	132/264 (50%)	207
5578-6411	*pk-1*	+L	277	10	Plxy pk-1	163/274 (59%)	350
6412-7339	*hr---*						
7340-7639	*11k*	−L	99	150	PlxyMNPV-11k	38/92 (41%)	60.5
7667-8791	*odv-e56*	+L	374	148	RaouMNPV odv-e56	289/371 (77%)	594
8840-11161	*ie-1*	−E	773	147	MaviMNPV ie-1	197/466 (42%)	348
11197-11626	*if-ac146*	+None	>143	146	AcMNPV orf146	79/138 (57%)	154

Results

Morphology of IrfaNPV

Electron microscope observation showed that IrfaNPV polyhedra are irregular in shape. Their sizes range from 0.9 to 1.9 μm in diameter, and the virion size is approximately 240 nm×50 nm. Each nucleocapsid rod was singly encapsulated to the virus lipoprotein envelope. The virus was therefore assigned to the single nucleocapsid NPV (SNPV) group of the baculoviridae (Fig. 1).

Cloning and sequencing of the major structural protein gene, *polyhedrin*

A plasmid library of *Eco*RI-*Xho*I fragments of the virus genome was built. A pair of primers corresponding to the conserved region of the baculovirus *polyhedrin* gene was used in PCR for screening of the library. A clone containing the *polyhedrin* gene of 11,626 kb was identified and sequenced. Thirteen ORFs homologous to reported baculovirus genes and a homologous repeat region (h) were identified within the sequenced region (Table 1). The BLAST results from the GenBank database showed that four of these genes, including *lef11*, *if-ac5*, *vp78*, and *if-ac*146, are best matched to their corresponding homologous genes from AcMNPV; three genes, including *if-ac38*, *pk-1*, and *11k*, are best matched to their homologs from *Plutella xylostella* multiple nucleopolyhedrovirus (PxlyMNPV); two genes (*39k* and *ie-1*) are best matched to those from *Maruca vitrata* MNPV (MaviMNPV); two (*ptp* and *lef2*) are best matched to their homologs from *Bombyx mori* NPV (BmNPV); *odv-e56* is best matched to its homolog from *Rachiplusia ou* MNPV (RaouMNPV); the *polyhedrin* gene is best matched to its homologue from *Antheraea pernyi* NPV (AnpeMNPV). All ORFs are best matched to homologs from members of Group I NPVs, with amino acid identities from 31% to 95%.

To investigate the relatedness of IrfaNPV to other baculoviruses, we compared the gene order in the *polyhedrin* region of IrfaNPV with the same region in several fully-sequenced Group I NPVs (Fig. 2). Three gene clusters are conserved between IrfaNPV and AcMNPV. The first cluster includes

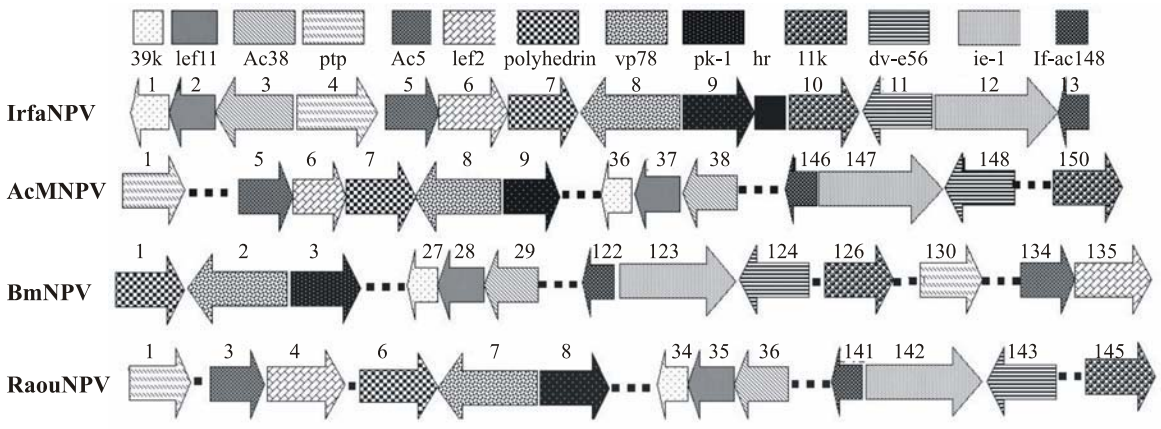

Fig. 2. Comparison of the gene organization of IrfaNPV with AcMNPV, BmNPV, and RaouMNPV.

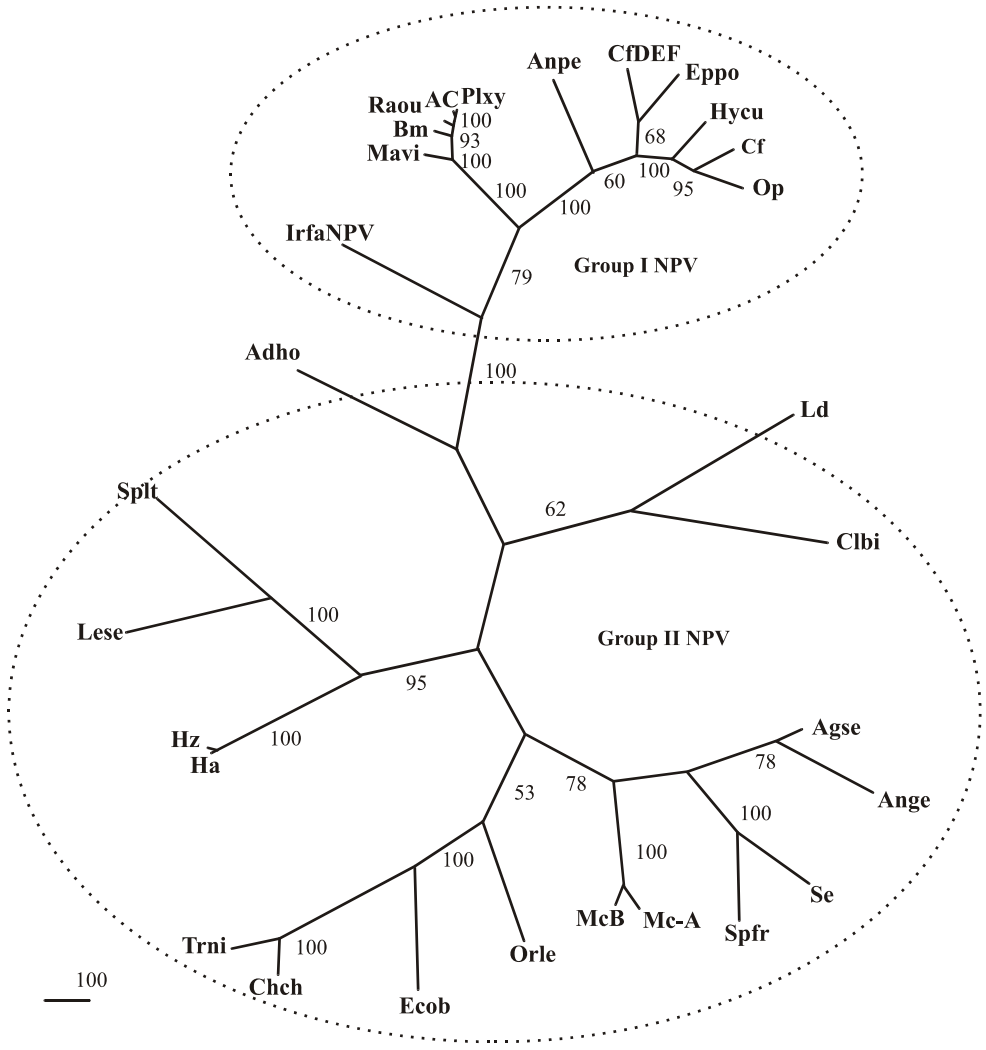

Fig. 3. A phylogenetic tree for nucleopolyhedroviruses based on the amino acid sequences of Lef2, polyhedrin, PK-1 and ie-1 joint genes. Statistical support (%) for each node was evaluated by bootstrap analysis with 1,000 replicates. The abbreviations of Baculovirus names and data sources (GenBank accession no.): Adho, *Adoxophyes honmai* NPV (NC_004690); Agse, *Agrotis segetum* NPV (NC_007921); Anpe, *Antheraea pernyi* NPV (NC_008035); Ange, *Anticarsia gemmatalis* NPV(NC_008520); Ac, *Autographa californica* MNPV (NC_001623); Bm, *Bombyx mori* NPV (NC_001962); CfDEF, *Choristoneura fumiferana* defective MNPV (NC_005137); Cf, *Choristoneura fumiferana* MNPV (NC_004778); Chch, *Chrysodeixis chalcites* NPV (NC_007151); Clbi, *Clanis bilineata* NPV(NC_008293); Ecob, *Ecotropis obliqua* NPV NC_008586); Eppo, *Epiphyas postvittana* NPV (NC_003083); Ha, *Helicoverpa armigera* SNPV (NC_003094); Hz, *Helicoverpa zea* SNPV (NC_003049); Hycu, *Hyphantria cunea* NPV (NC_007767); Lese, *Leucania separata* NPV (NC_008348); Ld, *Lymantria dispar* MNPV (NC_001973); Mc-A, *Mamestra configurata* NPV-A (NC_003529); Mc-B, *Mamestra configurata* NPV-B (NC_004117); Mavi, *Maruca vitrata* MNPV (NC_008725); Orle, *Orgyia leucostigma* NPV(NC_010276); Op, *Orgyia pseudotsugata* MNPV (NC_001875); Plxy, *Plutella xylostella* MNPV(NC_008349); Raou, *Rachiplusia ou* MNPV (NC_004323); Se, *Spodoptera exigua* MNPV (NC_002169); Spfr, *Spodoptera frugiperda* MNPV(NC_009011); Splt, *Spodoptera litura* NPV (NC_003102); Trni, *Trichoplusia ni* SNPV (NC_007383).

39k, *lef-11*, and *if-ac*38, in turn, with the same gene orientation in the two viruses. The second gene cluster includes *if-ac*5, *lef-5*, *polyhedrin*, *vp*78, and *pk*-1, with same gene orientations and positions in the genomes of the two viruses. The third cluster includes *11k*, *odv-e*56, *ie*-1, and *if-ac*146, with different orientations and positions in the genomes of the two viruses due to rearrangements and inversions of the gene cluster. Further investigation showed that all of the clusters were also found in the group I NPVs BmNPV and RaouNPV (Fig. 2). The degree of conservation of the clusters is evidence that IrfaNPV is more closely related to group I NPVs. However, the comparison of IrfaNPV with the Group I NPVs also showed extensive genomic translocations, as well as cluster inversions. Thus, the genomic organization of IrfaNPV is highly distinct.

Phylogenetic analysis

The amino acid sequences of four conserved baculovirus genes, including *ie-1*, *lef-2*, *pk-1*, and *polyhedrin*, were used for analysis of the phylogenetic relationship between IrfaNPV

and other NPVs. The results showed that IrfaNPV is in a phylogenetic position between Group I and Group II NPVs. However, it is more closely related to MaviNPV, RaouNPV, AcMNPV, PlxyMNPV, BmNPV, and other Group I NPVs (Fig. 3).

Infection of IrfaNPV in Tn cells

The proliferation of IrfaNPV in the non-host cell line Tn-5B1-4 was investigated using a quantitative PCR assay. The number of viral DNA copies increased from 24 to 96 h post-infection both in the cells and in the medium. These results indicate that IrfaNPV can infect TN-5B1-4 cells and form BVs that are released into the medium. However, the detected viral DNA copy number (BVs) in the medium was very low, only 1/1140 as high as that of AcMNPV BVs (Fig. 4). The AcMNPV final BV titer was 5.2×10^8 $TCID_{50}$/ml; thus the calculated relative titer of IrfaNPV was only about 4.6×10^5 $TCID_{50}$/ml. No typical NPV-infected cytopathic effects (CPE) were observed in the Tn-5B1-4 cells infected with IrfaNPV. The cells were alive and adhering to culture dish for more than one week and no polyhedra formed in the nucleus (Fig. 5). However, many vesicles appeared in the cytoplasm. The infection of Tn-5B1-4 cells with IrfaNPV showed that IrfaNPV is semi-permissive in these cells.

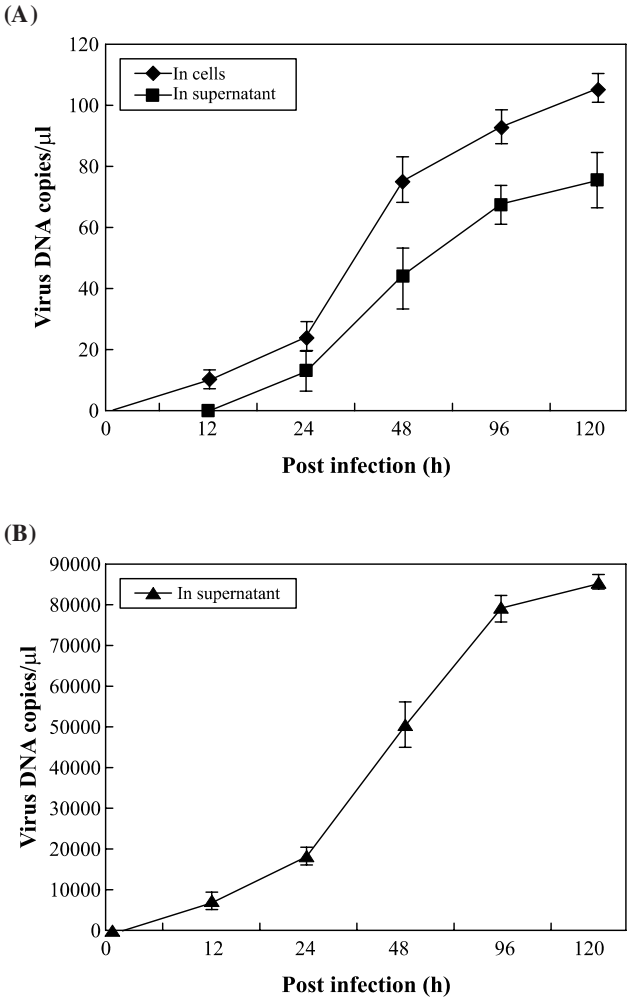

Fig. 4. IrfaNPV (A) and AcMNPV (B) replication curve in TN-5B1-4 cells.

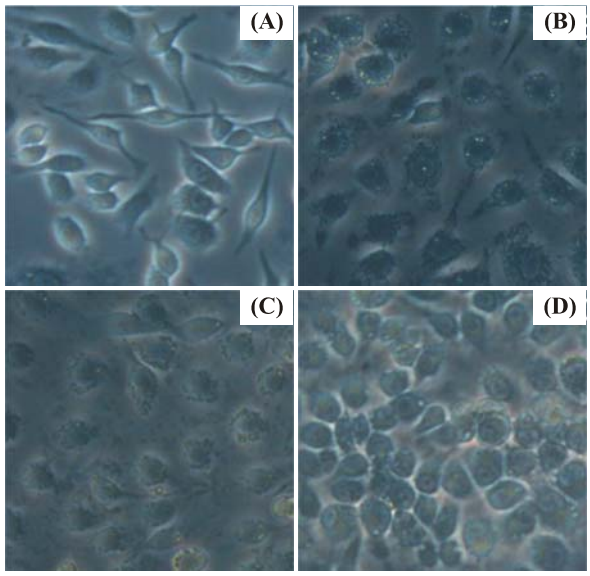

Fig. 5. Infection of TN-5B1-4 cells with IrfaNPV. (A) uninfected cells; (B) 24 h.p.i.; (C) 48 h.p.i.; (D) 96 h.p.i.

Biological activity

I. fasciata molts 5∼6 times in the larval stage and the young larvae (1st∼3rd instar) showed higher sensitivity to IrfaNPV. The 3rd instar larvae were used to evaluate the virus infectivity. A bioassay was designed to determine both lethal concentration of virus and lethal time of incubation. The results showed that mortality of *I. fasciata* larvae increased and the lethal time was shortened with increasing concentration of IrfaNPV. The time-dose-mortality analysis showed that the value of LC_{50} was 1.18×10^4 PIB/ml at 11 days post-inoculation, the 95% confidence limit was between 5.7×10^3 and 2.05×10^4 PIB/ml. At a concentration of 1×10^4, the median lethal time (LT_{50}) was 10.6 days.

Discussion

We report here the initial characterization of IrfaNPV, a new baculovirus recently isolated from tea slug moth larvae. An 11,626 bp genomic fragment containing the *polyhedrin* gene and 12 other genes was cloned and sequenced. BLAST results from GenBank showed that all of the 13 sequenced ORFs are well matched to homologs in Group I NPV members, including AcMNPV, BmNPV, PxlyMNPV, RaouMNPV, MaviMNPV, and AnpeNPV. Gene arrangement and phylogenetic analysis based on *polyhedrin*, *lef*-1, *pk*-1, and *ie*-1 genes further indicates that IrfaNPV is a member of the Group I NPVs. Genetic studies, including sequence analysis of the entire genome of IrfaNPV, will further help us to understand the diversity and evolution of baculoviruses.

A sustainable cell line will be a powerful tool for further genetic manipulation and multiplication of IrfaNPV. The

sequence comparison showed that IrfaNPV is closely related to AcMNPV and BmNPV. We tested several cell lines for susceptibility to IrfaNPV infection, including Tn-5B1-4, Sf9, and BmN. The infection of Tn-5B1-4 cells with IrfaNPV showed that IrfaNPV is semi-permissive in these cells, and it can proliferate in this non-host cell line at a low level. The mechanism of permissibility of insect cell lines to baculoviruses is complex. For example, AcMNPV can replicate strongly and produce polyhedra in Sf-9 cells, it was also permissive in 14 silkworm strains but nonpermisive in 17 silkworm strains (Guo *et al.*, 2005). In contrast, BmNPV can replicate and produce polyhedra in the BmN cell line, but it is weakly permissive in the Sf-9 cell line (Ikeda *et al*., 2001). BmNPV gene expression was dramatically reduced in Sf-9 cells inoculated with BmNPV. Experiments in which BmNPV DNA was transfected into Sf-9 cells suggested that this dramatic reduction in gene expression was not the result of poor attachment, penetration or uncoating of the BmNPV virion in Sf-9 cells (Iwanaga *et al*., 2004). The host range of AcMNPV can be expanded by replacing some amino acids of its DNA helicase with the corresponding residues from the BmNPV DNA helicase (Maeda *et al.*, 1993; Croizier *et al*., 1994). In addition to the *helicase* gene, some other baculovirus genes, such as AcMNPV *p35* (Clem and Miller, 1993), AcMNPV *ie0-ie1* (Kost *et al*., 2005), AcMNPV *hcf-1* (Lu and Miller, 1995), and *Lymantria dispar* MNPV *hrf-1* (Thiem *et al*., 1996; Du and Thiem, 1997), have been reported to be involved in the determination of host specificity (Volkman, 2007).

In conclusion, all sequence analysis demonstrated that IrfaNPV is a member of the Group I NPVs. The aforementioned characterization of the newly isolated virus will be helpful in the development of IrfaNPV as a biopesticide or an engineered pesticide.

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