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Morphological, Phylogenetic and Biological Characteristics of *Ectropis obliqua* Single-Nucleocapsid Nucleopolyhedrovirus

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The tea looper caterpillar, *Ectropis obliqua*, is one of the major pests of tea bushes. *E. obliqua* single-nucleocapsid nucleopolyhedrovirus (EcobSNPV) has been used as a commercial pesticide for biocontrol of this insect. However only limited genetic analysis for this important virus has been done up to now. EcobSNPV was characterized in this study. Electron microscopy analysis of the occlusion body showed polyhedra of 0.7 to 1.7 μm in diameter containing a single nucleocapsid per envelope of the virion. A 15.5 kb genomic fragment containing *Eco*RI-L, *Eco*RI-N and *Hin*dIII-F fragments, was sequenced. Analysis of the sequence revealed that the fragment contained eleven potential open reading frames (ORFs): *lef*-1, *egt*, 38.7*k*, *rr*1, *polyhedrin*, *orf*1629, *pk*-1, *hoar* and homologues to *Spodoptera exigua* multicapsid NPV (SeMNPV) ORFs 15, 28, and 29. Gene arrangement and phylogeny analysis suggest that EcobSNPV is closely related to the previously described Group II NPV. Bioassays on lethal concentration (LC_{50} and LC_{90}) and lethal time (LT_{50} and LT_{90}) were conducted to test the susceptibility of *E. obliqua* larvae to the virus.

Keywords: *Ectropis obliqua*, single-nucleocapsid nucleopolyhedrovirus, *polyhedrin*, phylogeny, bioassay

The *Baculoviridae* is a family of rod-shaped viruses with large, circular, covalently closed, double-stranded DNA genomes. Their DNA range in size from 81.7 kb for *Neodiprion lecontei* nucleopolyhedrovirus (NeleNPV) to 178.7 kb for *Xestia c-nigrum* GV (XecnGV). Two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV), have been recognized and distinguished 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 their molecular phylogenies (Zanotto *et al.*, 1993). Baculoviruses are specific pathogens for invertebrates, especially insects of the order Lepidoptera. They are being extensively studied for their usage in the expression of recombinant proteins and biological control of insect pests. The improvement of both applications requires a detailed knowledge of distinct baculovirus features and the extent of their diversity. In order to better understand the evolution of baculoviruses and the molecular mechanism behind baculovirus infection and replication, the sequencing of baculovirus genomes has been undertaken by a number of research groups (Zhang *et al.*, 2005).

Ectropis obliqua SNPV (EcobSNPV) is a singly embedded NPV pathogenic to the tea looper, *E. obliqua* Prout (Lepideptera: Geometride), one of the major pests of tea bushes in East Asia (Chen and Huang, 2001). The economic importance for the host of EcobSNPV makes it an important virus to study. It has been demonstrated that EcobSNPV is an effective and environmentally sound alternative to chemical insecticides (Hu *et al.*, 1994). The virus has been used to control the tea looper (Yin *et al.*, 2003) and was recently developed as a commercially available bio-pesticide agent with a registration number of LS20052031. About one thousand kilograms of EcobSNPV suspension at a concentration of 1×10^{10} PIB (polyhedral inclusion body)/kg is produced every year for controlling the tea looper in East China. Although the restriction maps of EcobSNPV have been assembled (Li *et al.*, 1983), little is known about its genetic analysis. In this study, we present the sequence analysis of a 15.5 kb region from the genome of EcobSNPV and compare it to the corresponding sequence and genetic organization from other baculoviruses. The larvae of *E. obliqua* were tested for their susceptibility to this vi-

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rus employing the lethal concentration (LC_{50}) and lethal time (LT_{50}) bioassay.

Materials and Methods

Virus and DNA

The virus used in this experiment was originally isolated from the tea looper, *E. obliqua* in Anhui Province in the People's Republic of China. The virus was propagated in 4th- instar larvae of *E. obliqua* 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.

Electronic microscopy

Midgut tissue of infected larvae was dissected and 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.

Restriction endonuclease (REN) analysis, PCR, and cloning

EcobSNPV genomic DNA was digested with various restriction endonucleases (*Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I and *Xho*I) and separated by 0.7% agarose gel electrophoresis (25 V, overnight) using standard techniques (Sambrook *et al.*, 1989). The size of restriction endonuclease DNA fragments was estimated from comparative mobility using a graphical method and *Hin*dIII λDNA markers. Most of the *Eco*RI-fragments smaller than 10 kb were cloned and sequenced (Table 1). Some fragments with sizes larger than 10 kb were analyzed with double enzyme digestions for confirmation of their sizes. The polymerase chain reaction (PCR) method was employed to check the relationship between *Eco*RI-L and *Eco*RI-N fragments. The following primers were used: 5'-CCG CTG TGG ACA AAC AC-3' (forward) and 5'-TCA AGT GTA GGC GAA GG-3' (reverse). PCR reaction was performed by standard protocols with annealing at 52°C. The PCR product was then gel-purified.

*Eco*RI-L (5.2 kb), *Eco*RI-N (3.8 kb), and *Hin*dIII-F (8.5 kb) fragments were cloned into plasmid pUC19, and the purified PCR product was cloned into T-easy vector. The recombinant plasmids were then transformed into *E. coli* TG1 using standard techniques (Sambrook *et al.*, 1989).

Nucleotide sequence analysis

Sequencing was carried out with the dideoxynucleotide chain terminating method using Sequenase™ Version 2.0 DNA sequencing kit (USB). The deduced amino acid sequences were compared with the updated GenBank/EMBL, SWISSPROT, and PIR databases using BLAST and FASTA programs (Pearson *et al.*, 1990; Altschul *et al.*, 1997). The data on other baculovirus genes compared in this paper were cited from GenBank or from published papers. The amino acid sequences were aligned based on a distance approach using CLUSTAL X version 1.81 with different gap opening and gap extension values. Following alignment, a phylogenetic tree was constructed for the combination of *polyhedrin*, *lef*-1, and *pk*-1 genes by the N-J method (PAUP* 4.0, beta 10 version) with the default settings of random break tie and the distance option of mean character difference. Statistical support for each node was evaluated by bootstrap analysis with 1,000 replicates. The tree was reformed by using TREEVIEW (V. 1.6.6), and the *Plutella xylostella* granulovirus (PxGV) sequence was used as an outgroup to estimate the position of the tree root.

The nucleotide sequence reported here was submitted to GenBank under the access number AF107100.

Bioassays

The occlusion bodies were suspended in sterile water at a concentration of 2 × 10^7 PIB/ml. A series of 10-fold dilutions was prepared from the OB stock solution. Six concentrations (20,000 PIB/ml, 2,000 PIB/ml, 200 PIB/ml, 20 PIB/ml, 2 PIB/ml, 0.2 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 EcobSNPV OB to second- instar larvae of *E. obliqua* 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 51-72 larvae per dose in triplicate. All analyses, including evaluation of virulence indices (LC50, LC90, LT50 and LT90), were performed using DPS software (Feng, 1998).

Results and Discussion

Transmission electron microscopy

Occlusion bodies (OBs) of EcobSNPV were observed in the infected midgut tissues of *E. obliqua* under a transmission electron microscope. The micrograph showed that EcobSNPV OBs were of irregular shape and ranged in size from 0.7 to 1.7 μm (1.15 ± 0.27

μm: mean ± SD) in diameter (data not shown). Multiple rod-shaped virions, measuring about 250 nm in length and 40 nm in width, were embedded in each OB with a single nucleocapsid packaged within the envelope of the virion (Fig. 1).

Restriction enzyme profile

Digests of the EcobSNPV genome with the restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I, and *Xho*I resulted in a total of 77 fragments larger than 1.0 kb (Fig. 2 and Table. 1). Based on estimated REN fragment sizes, the EcobSNPV genome was predicted to be about 127.7 kb. This is similar to the genomic size of 67.55-85.14×106 Da (102-129kb) reported by Li *et al* (1983). Fragments smaller than 1.0 kb were not figured into calculations. Southern blot analysis using *Bombyx mori* NPV (BmNPV) *polyhedrin* and *Autographa californica* MNPV (AcMNPV) *egt* probes indicated that overlapping fragments *Hin*dIII-F and *Eco*RI-N contained the EcobSNPV *polyhedrin* gene. *Hin*dIII-I and *Eco*RI-L fragments contained the *egt* gene (data not shown). Moreover, a PCR product was further sequenced to confirm the junction of *Eco*RI-L and *Eco*RI-N, which were separated from each other by a 465 bp *Eco*RI fragment. Therefore, the REN map of the whole fragment containing *Eco*RI-L, *Eco*RI-N, and *Hin*dIII-F was constructed by cloning the three fragments. It was sequenced to confirm the accurate localization and orientation of *polyhedrin*, *egt*, and other genes (Fig. 3).

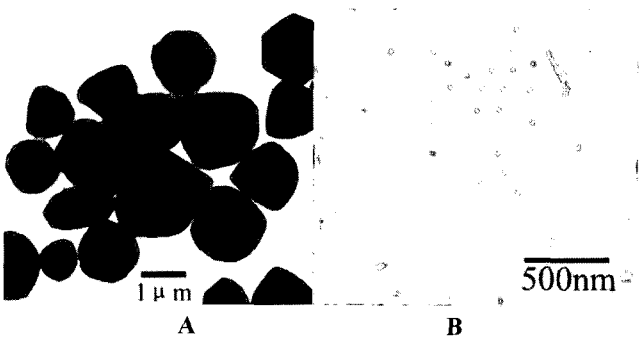

Fig. 1. Electron micrographs of polyhedra from *E. obliqua* single-nucleocapsid nucleopolyhedrovirus.

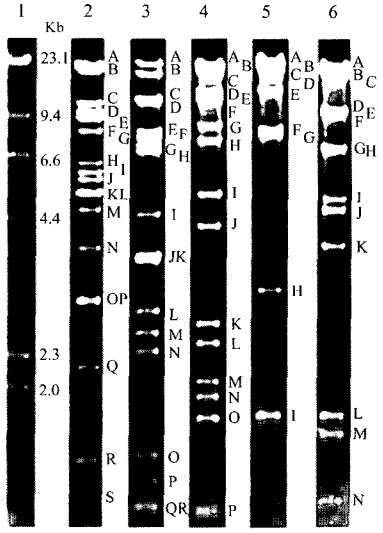

Fig. 2. Restriction endonuclease (REN) digestion fragments of EcobSNPV for *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I and *Xho*I. λ DNA digested with *Hin*dIII is included as a molecular size marker. The fragments were designated alphabetically starting with A for the largest fragment for each REN digest. Each visible band was assigned one or more letters depending on the number of fragments in each band. Lanes: 1, λDNA / *Hin*dIII; 2, *Eco*RI; 3, *Eco*RV; 4, *Hin*dIII; 5, *Pst*I; 6, *Xho*I.

Sequence determination and gene organization

The 15,528 bp EcobSNPV fragment was sequenced. Eleven ORFs homologous to baculovirus proteins were identified within the sequenced region: a late expression factor 1 gene (*lef-1*), a homologue to the *Spodoptera exigua* multicapsid NPV (SeMNPV) ORF15 (*Eo-se*15), an ecdysteroid UDP-glucosyltransferase gene (*egt*), a homologue to the SeMNPV ORF28 (*Eo-se*28), a homologue to the SeMNPV ORF29 (*Eo-se*29), a 38.7 kD protein (38.7*k*), a ribonucleotide reductase (*rr*1), a

Table 1. Estimated sizes of EcobSNPV DNA restriction fragments (kb)

Fragment	*Eco*RI	*Eco*RV	*Hin*dIII	*Pst*I	*Xho*I
A	22	25	23	27	22
B	20	19	20	25	20
C	11	12	18	19.5	18
D	10	12	13.3	19.5	9.9
E	9.163*	8.3	10.5	14.0	9.8
F	8.097*	7.8	9.911*	8.5	9.6
G	7.976*	7.0	8.6	8.3	7.0
H	6.4	6.8	7.8	3.07	7.0
I	6.201*	4.45	5.152*	1.75	5.0
J	6.1	3.70	4.20	1.75	4.80
K	5.209*	3.60	2.70		3.83
L	5.152*	2.85	2.45		1.75
M	4.720*	2.54	2.08		1.65
N	3.831*	2.36	1.95		1.22
O	3.057*	1.48	1.72		
P	3.002*	1.33	1.16		
Q	2.094*	1.17			
R	1.45	1.17			
S	1.20				
Total	134.5	121.4	132.5	128.4	121.5

* These fragments were cloned and sequenced.

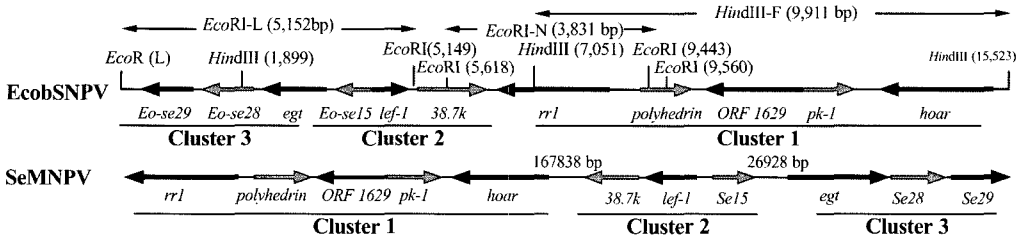

Fig. 3. Gene organization of the *egt-polh* region of EcobSNPV and its comparison with the corresponding regions of SeMNPV. The arrows represent the ORFs and point in the directions of their transcription.

major occlusion protein (*polyhedrin*), a virus replication-essential protein (*orf*1629), a protein kinase (*pk*-1) and a homologue of the HzSNPV ORF4 (*hoar*) (Fig. 3).

To investigate the relatedness of EcobSNPV to other baculoviruses, we compared the gene order in the *egt-polh* region of EcobSNPV with fully-sequenced lepidopteran NPVs. Fig. 3 shows a representative comparison of the gene arrangements between EcobSNPV and SeMNPV. The genetic organization and the putative map of transcripts of this region indicated that a core gene cluster of three genes, *polyhedrin, orf*1629, *and pk*-1, was present in both aforementioned baculoviruses. Further study indicated that the set of these three genes has remained in the same relative position (orientation may be different) in all of the sequenced genomes from lepidopteran NPVs with the exception of *Adoxophyes honmai* NPV (AdhoNPV). In this virus, *alk-exo* and another ORF are inserted between *polyhedrin* and *orf*1629. The fact that these genes are found in the same relative position in most lepidopteran NPVs supports the use of the *polyhedrin* gene as point of reference to orient baculovirus physical maps. This also indicates that there may be some physical constraint preventing them, or at least these DNA sequences, from being separated. The gene map also suggested that three gene clusters are conserved between EcobSNPV and SeMNPV. The first cluster includes *rr*1, *polyhedrin*, *orf*1629, *pk-1,* and *hoar*, in turn, with same gene orientation and genomic position in these two viruses. The second gene cluster includes *38.7k*, *lef*-1, and *Se*15 homologues. The third cluster includes *egt*, *Se*28, and *Se*29 homologues, with different orientation and position in the genomes between these two viruses, due to rearrangements and inversions of the gene cluster. Further investigation showed that all of the aforementioned three clusters were also found in *Mamestra configurata* NPV-A (MacoNPV-A) and MacoNPV-B genomes. In addition, the following clusters were found to be present in other group II NPV genomes: clusters 1 and 2 within *Chrysodeixis chalcites* NPV (ChchNPV) and *Trichoplusia ni* SNPV (TrniSNPV), clusters 2 and 3 within AdhoNPV, cluster 2 within *Spodoptera litura* MNPV (SpltMNPV)

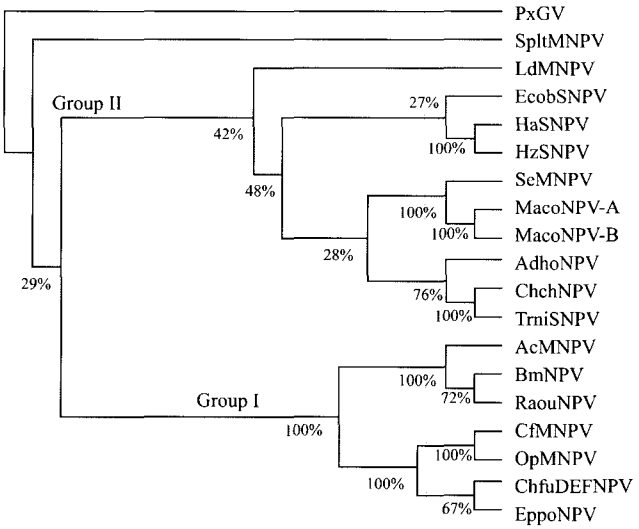

Fig. 4. Phylogenetic analysis using the predicted amino acid residues from *polyhedrin*, *lef*-1 and *pk*-1. The tree was constructed using N-J method (PAUP* 4.0, beta 10 version) and branch numbers represent bootstrap probabilities (%) of 1,000 replicates. The tree was reformed by using TREEVIEW (V. 1.6.6), and the *Plutella xylostella* granulovirus (PxGV) was used as an outgroup.

and cluster 3 within *Lymantria dispar* MNPV (LdMNPV). Surprisingly, none of the above three clusters could be found in Group I NPVs like AcMNPV, BmNPV, *Orgyia pseudotsugata* MNPV (OpMNPV), *Epiphyas postvittana* NPV (EppoNPV), and others. The conserved clusters gave evidence that EcobSNPV is more closely related to group II NPVs.

Phylogenetic position within NPVs

Polyhedrin is the most extensively studied baculovirus gene and usually used to understand the phylogenetic relationships of baculoviruses. Sequence alignment for amino acids encoded by 20 NPV *polyhedrin* genes indicates that EcobSNPV *polyhedrin* shares a high degree of homology with 17 other lepidopteran NPVs, with identities of 82-94.3%. As an outgroup in Fig. 4, PxGV *granulin* shares 54.1% identity with EcobSNPV *polyhedrin*. In contrast, *polyhedrin* genes from two fully sequenced hymenopteran NPVs, *Neodiprion le-*

contei NPV (NeleNPV) and *Neodiprion sertifer* NPV (NeseNPV) share very low identities (44.7% and 47.2%, respectively) with that of EcobSNPV (data not shown), indicating that hymenopteran NPV (NeleNPV and NeseNPV) may have existed before the divergence of lepidopteran NPV and GV (Lauzon *et al.*, 2004; Garcia-Maruniak *et al.*, 2005).

The phylogenetic tree (Fig. 4) of NPVs based on the combined sequences of *polyhedrin*, *lef*-1, and *pk*-1 indicated that EcobSNPV was most closely related to *Helicoverpa armigera* SNPV (HaSNPV) and *Helicoverpa zea* SNPV (HzSNPV). It also appeared that the relationships between SeMNPV, MacoNPV, AdhoNPV, ChchNPV, TrniSNPV, LdMNPV, and EcobSNPV were closer. It is more distantly related to other lepidopteran NPVs, such as AcMNPV, BmNPV, OpMNPV, *Choristoneura fumiferana* MNPV (CfMNPV) and EppoNPV. This evidence strongly suggested that EcobSNPV is a member of the Group II NPVs. Based on DNA polymerase gene sequence alignments, Bulach *et al.* (1999) described LdMNPV as a Group II NPV. Three Group II NPV subclades were classified as A, B, and C. Further phylogenetic analysis of *polyhedrin* gene suggested that EcobSNPV belonged to subgroup II-C.

Although *polyhedrin* is still considered a reasonable marker for identification of its neighbors, Herniou *et al.* (2003) and Lange *et al.* (2004) argued that it might not be the best baculovirus gene for phylogenetic studies because *polyhedrin* phylogenies often disagree with other gene phylogenies. While other phylogenetic analyses consistently group AcMNPV and BmNPV together, phylogenies based on *polyhedrin* have AcMNPV as a sister group to the rest of the group I NPVs (Herniou *et al.*, 2003). Phylogenies based on combined sequences of shared genes have been found to be more robust than those based on the sequences of individual genes (Herniou *et al.*, 2001; 2003). Thus in this study, we selected *polyhedrin*, *lef*-1, and *pk*-1 gene sequences to construct a baculovirus phylogenetic tree based on their presence in all sequenced NPV and GV genomes. Of course, the concatenation of more genes in common between the genomes of interest may provide more reliable information for the phylogenesis after the genome of EcobSNPV is fully sequenced.

Biological activity

EcobSNPV was evaluated for its infectivity in second-instar larvae of *E. obliqua*. A bioassay was designed to determine both lethal concentration of virus and lethal time of incubation. The results showed that mortality of *E. obliqua* larvae increased and the lethal time was shortened with increasing concentration of EcobSNPV. When the second instar larvae were treated with high concentrations of EcobSNPV between 20,000 and 2,000 PIB/ml, all died within 10 to 14 days. However, only 30.6% of the larvae died at 18 days post-inoculation when they were treated with a concentration of 0.2 PIB/ml. The time-dose-mortality analysis showed that the value of LC_{50} was $10^{4.09}$ PIB/ml at 7 days post-inoculation and $10^{0.8}$ PIB/ml at 14 days post-inoculation (Table 2). At a concentration of 2,000 PIB/ml, LT_{50} was 8.5 days and LT_{90} was 10.7 days (Table 3).

In conclusion, we report here the initial characterization of EcobSNPV, a baculovirus that infects the tea looper, *E. obliqua* Prout, an insect that poses important economic concerns. A 15.5 kb genomic DNA sequence was analyzed and 11 genes were identified. The genetic organization and transcription profile of the EcobSNPV *egt-polh* region, like the sequence alignment of other baculoviruses, showed a considerable degree of similarity to SeMNPV, MacoNPV-A, MacoNPV-B, and other Group II NPVs. Baculovirus

Table 2. LC_{50} and LC_{90} values for second instar tea looper exposed to EcobSNPV at different time post-inoculation

Days	LC_{50} ± S.E.	LC_{90} ± S.E.
3	7.33 ± 0.49	8.70 ± 0.53
4	6.11 ± 0.30	7.48 ± 0.35
5	5.55 ± 0.24	6.92 ± 0.29
6	4.68 ± 0.16	6.04 ± 0.21
7	4.09 ± 0.13	5.45 ± 0.17
8	3.54 ± 0.11	4.91 ± 0.14
9	3.10 ± 0.10	4.47 ± 0.12
10	2.15 ± 0.10	3.52 ± 0.10
11	1.80 ± 0.11	3.17 ± 0.10
12	1.39 ± 0.12	2.77 ± 0.10
13	1.08 ± 0.12	2.45 ± 0.10
14	0.80 ± 0.12	2.17 ± 0.10

Table 3. LT_{50} and LT_{90} values for EcobSNPV against second instar tea looper at different concentrations

Concentration (PIB/ml)	LT_{50}	LT_{90}
20,000	6.6	9.3
2,000	8.5	10.7
200	9.8	11.6
20	12.3	-

phylogeny has served as a framework for placing data from the comparison of baculovirus genomes into an evolutionary context (Herniou *et al.*, 2003). Molecular genetic studies, including sequence analysis of the entire genome of EcobSNPV, will further help us to understand the diversity and evolution of baculoviruses. It is well known that baculovirus infection is one of the factors that cause susceptible insect populations to fluctuate in the field. These extensive bioassays will be helpful for the development of EcobSNPV as a biopesticide.

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References

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programes. *Nucleic Acids Res.* 25, 3389-3402.

Blissard, G.W., B. Black, N. Crook, B.A. Keddie, R. Possee, G. Rohrmann, D. Theilmann, and L. Volkman. 2000. Family Baculovidae. In Virus Taxonomy. p. 195-202. Seventh Report of the internation Committee on Taxonomy of Viruses. *In* van Regenmortel *et al.* (eds.), Academic Press, San Diego, California.

Bulach, D.M., C.A. Kumar, A. Zaia, B. Liang, and D.E. Tribe. 1999. Group II nucleopolyhedrovirus subgroups revealed by phylogenetic analysis of polyhedrin and DNA polymerase gene sequences. *J. Invertebr. Pathol.* 73, 59-73.

Chen, Y.G. and M.D. Huang. 2001. Biological control progress of *Ectropis obliqua* Prout. *Natural Enemies of Insects* 23, 181-184.

Feng, M.G., C.L. Liu, J.H. Xu and Q. Xu. 1998. Modeling and biological implication of the time-dose-mortality data for the entomophthoralean fungus, Zoophthora anhuiensis on the green peach aphid, *Myzus percisae. J. Invertebr. Pathol.* 72, 246-251.

Herniou, E.A., T. Luque, X.W. Chen, J.M. Vlak, D. Winstanley, J.S. Cory, and D.R. O'Reilly. 2001. Use of whole genome sequence data to infer baculovirus phylogeny. *J. Virol.* 75, 8117-8126

Herniou, E.A., J.A. Olszewski, J.S. Cory, D.R. O'Reilly. 2003. The genome sequence and evolution of baculoviruses. *Annu. Rev. Entomol.* 48, 211-234.

Hu, C., J.Q. Zhu, G.Y. Ye and J. Hong. 1994. *Ectropis obliqua* Prout, a serious geometrid pest of tea bush in East China. p. 217. Shanghai Scientific and Technical Publishers, Shanghai, China.

Lange, M., H. Wang, Z. Hu, and J.A. Jehle. 2004. Towards a molecular identification and classification system of lepidopteran-specific baculoviruses. *Virology* 325, 36-47.

Li, M.T., P.H. Dai, and J.H. Xin. 1983. The restriction endonuclease digestion maps and electron microscopy analysis of *Ectropis obliqua* NPV DNA. Microorganism dissertation. p. 119-123. Science Press, China.

O'Reilly, D.R., L.K. Miller, and V.A. Lucknow. 1992. Baculovirus expression vectors: A Laboratory Manual. Oxford University Press, New York.

Pearson, W.R. 1990. Rapid and sensitive sequence comparision with FASTP and FASTA. *Methods Enzymol.* 183, 63-98.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Yin K.S., H. C. Chen, M.J. Tang, H.W. Guo, Q. Xiao, and H.M. Yao. 2003. Studies on application techniques of EoNPV preparations. *Virologica Sinica.* 18, 492-495.

Zanotto, P.M., B.D. Kessing, and J.E. Maruniak. 1993. Phylogenetic interrelationships among baculovirus: evolutionary rates and host associations. *J. Invertebr. Pathol.* 62, 147-164.

Zhang, C.X., X.C. Ma, and Z.J. Guo. 2005. Comparison of the complete genome sequences between C1 and G4 isolates of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus. *Virology* 333, 190-199.