Russian Research Institute for Silviculture and Mechanization of Forestry
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RECENT ADVANCES IN THE RESEARCHES AND APPLICATION OF VIRUSES AND ENTOMOPHAGES IN FOREST HEALTH PROTECTION

Pushkino-Beijing
2018

Recent advances in the researches and application of viruses in forest health protection and entomophages / Editors Yu.I. Gninenko and Zhang Yong-an. – VNILLM : Pushkino-Beijing, 2018. – 142 p.

ISBN 978–5–94219–226–6

Editorial foreword

This collection of papers is the third joint publication of Chinese and Russian authors. Encouraging that colleagues from other states join Chinese and Russian authors.

The first 2 publications mostly focused on virus preparation development and applications in forest protection and this publication maintaining virus priority pays more than usual attention to other areas of biological forest protection.

Baculovirus application is a key area in protection of tree and shrub species. Being one of the most environmental microbiological forest protection areas it enables reliable protective impact. Significantly baculovirus related work is rather wide spread in China and began to renew in Russia. The already developed virus preparations are applied in big areas in China providing forest protection against hazardous needle and leaf eating pests.

Recently a new highly efficient agent neovir based on red pine sawfly nuclear polyhedrosis was developed in Russia. It can successfully replace the well known virus preparation virin-diprion widely applied in pine protection against red pine sawfly larvae.

However other biological protection areas are under development. The classical biological procedure namely entomophage application is getting higher demand in some states in particular due to arising forest protection challenges by new invasive organisms.

We believe that this third Russian-Chinese collection of papers will find its readers and serve to develop forest protection biological area not only in China and Russia but other states as well.

Yu. Gninenko, Zhang Yongan

Предисловие редакторов

Третье совместное издание научных статей Китайских и Российских авторов. Вдохновляет то, что к Китайским и Российским авторам присоединились коллеги из других стран.

Две первые работы в основном сконцентрированы на разработке и применении вирусных препаратов в защите леса, а данное издание, уделяя приоритет вирусам, более чем обычно заостряет внимание на других направлениях биологической защиты леса.

Применение бакуловирусов является основным направлением в защите древесных и кустарниковых пород. Являясь одним из наиболее экологических направлений микробиологической защиты леса, оно обеспечивает надёжное защитное воздействие. Существенная связанная с бакуловирусами работа проводится довольно широко в Китае и начала восстанавливаться в России. Уже разработанные вирусные препараты применяются на больших площадях в Китае, обеспечивая защиту леса от вредных хвое- и листогрызущих вредителей.

Недавно в России был разработан новый высокоэффективный препарат – неовир – на основе ядерного полиэдроза красноголового соснового пилильщика. Он сможет успешно заменить хорошо известный вирусный препарат вирин-диприон, широко применяемый в защите сосны от личинок красноголового соснового пилильщика.

Тем не менее, продолжаются исследования в других направлениях биологической защиты леса. В некоторых странах растёт спрос на классический биологический метод, а именно – применение энтомофагов, в частности в связи с возрастающими проблемами защиты леса из-за новых инвазивных организмов.

Мы полагаем, что данный 3-й Российско-Китайский сборник научных работ найдёт своих читателей и послужит развитию направления биологической защиты леса не только в Китае и России, и в других странах.

Ю. Гниненко, Чжан Йонган

Содержание

FIELD TESTING A CHINESE, JAPANESE AND THE REGISTERED STRAIN OF THE NORTH AMERICAN GYPSY MOTH VIRUS AGAINST THE CHINESE STRAIN OF THE ASIAN GYPSY MOTH IN HUHHOT, INNER MONGOLIA, CHINA

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摘要：2004-2006年三次分别对三株不同地理位置分离到的舞毒蛾核型多角体病毒在分布于内蒙古的舞毒蛾中国亚种上的生物活性进行了林间测定。其中来自中国的舞毒蛾核型多角体病毒毒株(LdMNPV-H)致病力最高，LC50 和 LC95 分别为6.5 OBs/μL和742 OBs/μL。北美舞毒蛾核型多角体病毒毒株(LdMNPV-D)的致病力中等，LC50和LC95 分别为31.5 OBs/μL和4 093 OBs/μL。日本舞毒蛾核型多角体病毒毒株(LdMNPV-J)的致病力最低，但LC50为14.7 OBs/μL，介于中国和北美分离株之间，而LC95最高为11542 OBs/μL。从中国舞毒蛾亚种对三株不同分离株病毒剂量的反应来看， LdMNPV-H 和LdMNPV-D的敏感性高于LdMNPV-J。从对病毒的反应时间来看，LdMNPV-J病毒的毒力明显高于其它两株，但是病毒剂量也高于其它两株，而致死50%幼虫的死亡时间要比其它两株病毒短，LdMNPV-J为11.1天， LdMNPV-H和LdMNPV-D毒株分别为13.5和14.7天。2005年喷洒LdMNPV-D病毒后立刻下大雨对舞毒蛾幼虫的死亡率造成明显的影响。

关键词：杆状病毒；地理种；毒力；致病性；亚洲舞毒蛾

ПОЛЕВЫЕ ИСПЫТАНИЯ КИТАЙСКИХ, ЯПОНСКИХ И ЗАРЕГИСТРИРОВАННЫХ СЕВЕРОАМЕРИКАНСКИХ ВИРУСОВ НЕПАРНОГО ШЕЛКОПРЯДА ПРОТИВ АЗИАТСКОЙ ФОРМЫ НЕПАРНОГО ШЕЛКОПРЯДА В ХУХОТО, ВНУТРЕННЯЯ МОНГОЛИЯ, КИТАЙ

Показана биологическая эффективность штаммов ядерного полиэдроза непарного шелкопряда, полученная из разных популяций непарного шелкопряда во время испытаний в 2004-2006 гг., проведенных на гусеницах азиатской формы непарного шелкоряда в провинции Внутренняя Монголия (Китай). Китайский штамм вируса оказался более эффективным, тогда как штамм из Северной Америки имел среднюю эффективность. Наименьшая смертность гусениц отмечена при использвоании японского штамма.

Однако время достижения 50%-ной смертности гусениц оказалось наименьшим для японского изолята (11.1 д.), тогда как для китайского изолята оно составило 13.5 дн., а для североамериканского – 13.5 и 14.7 дн.

Дождь, прошедший в 2005 г. сразу после проведения опрыскивания, существенно снизил уровень смертности гусениц.

Ключевые слова: бакуловирус, азиатский непарный шелкопряд, вирулетность, патогенность, географические изоляты.

ABSTRACT: The biological activity of three geographic isolates of the gypsy moth nucleopolyhedrovirus (LdMNPV) was evaluated in the field against larvae of the Chinese strain of the Asian gypsy moth in Inner Mongolia, P.R. China, in 2004, 2005 and 2006. The Chinese strain of the virus (LdMNPV-H) was the most pathogenic of the isolates tested, having the lowest LC_{50} and LC_{95} (6.5 and 742 OBs/µL, respectively). The North American strain of the virus (LdMNPV-D) was moderately pathogenic, with LC_{50} of 31.5 OBs/µL and LC_{95} of 4 093 OBs/µL. The Japanese strain (LdMNPV-J) was the least pathogenic, it had the second lowest LC_{50} (14.7 OBs/µL) but highest LC_{95} (11 542 OBs/µL). The slopes of the dose-response regression lines for Chinese gypsy moth larvae to the three virus isolates indicated that the Chinese strain larvae were more homogenously susceptible to LdMNPV-H and LdMNPV-D than to LdMNPV-J. Time-response data showed that LdMNPV-J was significantly more virulent than the other two isolates, but at a much higher dose, causing 50% mortality in the shortest period of time (11.1 d), followed by LdMNPV-H and LdMNPV-D (13.5 and 14.7 d, respectively). Rainfall immediately after the application of LdMNPV-D in 2005, as expected, significantly reduced gypsy moth larval mortality.

Key words: baculovirus; geographic strain; virulence; pathogenicity; Asian gypsy moth.

The gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), is a native defoliator in both Europe and Asia. Since its intentional importation from Europe and accidental escape near Boston, Massachusetts, in 1868 or 1869, the European gypsy moth has spread over a wide area of North America. It now occurs throughout the northeastern United States from central Wisconsin to North Carolina, and into Canada in the southern Maritimes, Quebec and central Ontario (Régnière et al. 2009), causing significant defoliation of forested areas (McManus and McIntyre 1981, Leonard 1981, Elkinton and Liebhold 1990). The gypsy moth is also a major pest in forested areas in China and Japan (Pogue and Schaefer 2007).

Recent taxonomic reclassification of the family Lymantriidae, based on morphological, genetic and molecular characteristics, prompted a review of the status of the genus *Lymantria*. Following a thorough study of members of this genus, Pogue and Schaefer (2007) proposed some nomenclatural changes for the common and scientific names of gypsy moth. In this new nomenclature, the species *L. dispar* (subgenus *Porthetria*) is divided into three subspecies, and three new species. According to this classification system, the term “gypsy moth” (*L. dispar dispar* (L.)) now refers specifically to the flightless gypsy moth native to Europe and introduced into North America (EGM), as well as the nearly wingless gypsy moth occurring in India. The “Asian gypsy moth” (*L.d. asiatica* Vnukovskij) (AGM) are those *L. dispar* that have winged females capable of flying, that occurs from the eastern slopes of the Ural Mountains in Russia to the Pacific Ocean, in the northern two-thirds of China and Korea. The Japanese gypsy moth (*L.d. japonica* (Motschulsky)) (JGM), refers specifically to the gypsy moth found only on the Japanese islands of Honshu, Shikoku, Kyushu, and parts of southern and western Hokkaido.

Females of the JGM, AGM, and many females that are hybrids between the Asian and European strains, are able to fly, increasing their dispersal capability (Reineke and Zebitz 1998, Keena et al. 2001). The AGM also has a much broader host range, including both deciduous and coniferous trees, and therefore, poses a greater threat to North American forests than the European strain (Baranchikov 1988, Walsh 1993). Timber losses in North America, to date, have been caused solely by the EGM, because the AGM, luckily, has not yet become established in North America (Krcmar-nozic et al. 2000), i.e. eradication attempts have so far been successful. Should the AGM be allowed to become established in North America, its ability to feed on a wider range of host plants and the ability of female AGM to fly may allow it to spread much more rapidly than the EGM, and likely leading to more devastating economic losses in forestry.

During the past 140 years since its escape and establishment in North America, many strategies for eradication, control and suppression of the EGM have been developed and tried, including the introduction of the fungal pathogen *Entomophaga maimaiga* Humber, Shimazu and Soper (Zygomycetes: Entomophthorales), from Japan, to slow its spread (McManus and McIntyre 1981, Hajek 1999, Sharov et al. 2002). The use of a nucleopolyhedrovirus (NPV) that occurs in gypsy moth populations is an important regulator in high-density populations (Podgwaite 1981). It has been developed as a biopesticide for control of gypsy moth and was first registered by the US Environmental Protection Agency in 1978 under the name Gypchek. In addition, researchers in Canada and the former U.S.S.R. also successfully developed this baculovirus as a bioinsecticide for gypsy moth control (Webb et al. 1989, Cunninghan et al. 1991, Podgwaite et al. 1992, Cunningham 1995). The virus products developed for use in the United States and Canada were derived from the same NPV isolate (Cunningham 1995), but registered in these countries under different names (Gypchek in the United States (Reardon et al. 1996) and Disparvirus in Canada (Cunningham et al. 1991)). The U.S.S.R product, VIRIN-ENSh, was developed from a different viral isolate (Dougherty 1983) that has similar biological activity (Shapiro 1983). In preparation for the potential establishment of the AGM in North America, it is highly desirable to examine the control capabilities of existing biopesticides and to increase the potential number of pest management tools currently available to forest managers.

We report here on the biological activity of the three geographic isolates of LdMNPV, previously tested in the laboratory (unpublished data), in the field against the Chinese strain of Asian gypsy moth (AGM-C) in Inner Mongolia Autonomous Region, People's Republic of China (PRC). We conducted this study to determine the field activity of LdMNPV-D, the active ingredient in the virus products registered to control gypsy moth in North America, against the AGM-C. We also wanted to compare the biological activity of LdMNPV-H and LdMNPV-J to compare their efficacy to LdMNPV-D, and to speed up the eventual selection of the most virulent strain to be developed into a viral biopesticide for the control of the AGM, should it become established in North America and the need arise for an additional biopesticide other than the currently registered product. Lastly, we wanted to determine the efficacy of these three virus products when applied against AGM-C on a native host tree in China, and compare these results with those of previous laboratory studies with the same three virus products.

Materials and Methods

Experimental plot. The experiments were conducted in the research area of the Inner Mongolia Agricultural University, located in Huhhot, Inner Mongolian Autonomous Region, PRC. Our study plot (50 m x 12 m) was located in northeast corner of the 30 ha university research area, and was surrounded by apple (*Malus* sp.) and cherry (*Prunus* sp.) trees in east, corn in the north and south, and grass in the west. Our experimental plot was weeded, as required, during the 3 years of the experiment.

Host tree. *Populus cathayana* Rehder (common name in China is "qing yang"), one of the hosts of AGM in China, was the host tree used in this experiment. *P. cathayana* is one of the most common and widely distributed species of *Populus* in central China [its range includes southern Gansu, Hebei, Lianong, Nei Mongol (Inner Mongolia), eastern Qinghia, Shaanxi, Shanxi, Sichuan, Yunnan and Zhejiang Provinces] (Fang et al. 2008, Chao et al. 2009), where it grows along river valleys at elevations between 800 to 3000 m. Mature trees can reach a height of 30 m (Weisgerber and Han 2001).

Rooted cuttings. To prepare for the field tests, at the end of April 2003, five rows (1.5 m apart) of 30 pits, each 25-30 cm deep, and spaced 2 m apart, were dug in the study plot. To obtain the rooted cuttings, a total of 600 twigs (each approximately 50 cm long) were cut from five adjacent mature *P. cathayana* trees located near our experimental plot, part of a row of trees initially propagated in 1988 from cuttings taken from a single, naturally growing tree. The cuttings were propagated by planting four of these twigs into each of the pits. The twigs were watered at planting, and once or twice a week thereafter, depending on weather conditions. About 95% of the cuttings rooted and survived, resulting in some pits containing only three, rather than four rooted cuttings. On June 24 in 2004, rooted poplar cuttings that had grown to more than 60 cm were cut back to a height of about 50-60 cm (to fit into the pre-constructed cages). Pruning was done in such a way as to ensure that the three or four rooted cuttings in each pit retained enough leaves (a total of approximately 100 leaves), for treatment with distilled water (control) or one of the three concentrations of the three LdMNPV isolates.

Cages. Frames for each of the 72 cages (75 cm x 75 cm x 80 cm high) were constructed from steel construction rods (0.8 cm diameter). The pieces were welded together at the eight corners to create the frame. Fine nylon mesh, mesh size ca. 0.3 mm x 0.3 mm, was sewn to two opposing sides and across the top of the steel frame. Two other pieces of mesh were attached along the top edge of the uncovered sides, and Velcro® zippers were sewn on each of the corners of each cage to make flaps that could be opened to allow checking larval mortality. One cage was installed around each of the pits containing three or four of the rooted poplar cuttings. The ground inside and outside the cages was cleared of vegetation, and the rooted poplar cuttings were thoroughly searched for naturally-occurring insects, which were removed before placing the cages and introducing the test insects (30 larvae in each cage in 2004 and 2006, 20 larvae in 2005). There were no naturally-occurring gypsy moth larvae in our experiment plot or the surrounding the experimental research area, only some *Carabus brandtis* Faldermann adults, larvae in the ground and some unidentified ants.

Test insects. AGM-C egg masses (between 0.5-1.0 kg, i.e. 1200-2500 egg masses) were collected in the spring of each year of the experiment (March 16, 2004, March 10, 2005 and March 25, 2006) from the He-Ling Forest Farm in Inner Mongolia Autonomous Region, where the gypsy moth populations were feeding on Prince Rupprecht's larch, *Larix principis-rupprechtii* Mayr. This infestation had

been in progress for 3 years and was in its increasing phase in 2004, with the infestation reaching its peak in 2005 and starting to decline in the fall of 2006. Each year, the collected egg masses were stored under refrigeration at 5°C for approximately 2-3 months before use.

AGM-C eggs were surface decontaminated prior to being set up for rearing. Thirty groups of eggs, each containing approximately 500 eggs, were placed into cheesecloth and immersed into 1% bleach solution for 30 seconds and then rinsed for 1 min each of five times in distilled water, and allowed to dry on filter paper at room temperature. The decontaminated eggs were put into 6 oz. containers (OZ6-XE6) with DS306 cardboard lids (Sweetheart Cup Co., Owing Mills, MD), 500 eggs in each of 30 cups, and reared at 25ºC, 50%RH, and 16L:8D light-dark photoperiod.

The hatched larvae were transferred to new 6 oz. containers containing a modified artificial gypsy moth diet (Bell et al 1981, Odell et al 1997) and reared (100 larvae in each of 150 cups, total of 15,000 larvae) under the same conditions as above until just before placing them in the cages. On the day before the larvae were placed on the treated poplar trees, newly molted (less than 24 h) second-instar larvae were transferred into clean 6 oz. fluted containers containing no diet (30 larvae per cup in 2004, 20 larvae per cup in 2005 and 30 larvae per cup in 2006) and starved for 24 h before being placed onto the leaves of the treated rooted popular cuttings in the cages.

Virus strains. Three geographic isolates of *L. dispar* NPV were provided by Natural Resources Canada, Canadian Forest Service (CFS), Pacific Forestry Centre (PFC), to the Forestry College, Agricultural University of Inner Mongolia for testing. Two of the virus isolates were collected from field populations of gypsy moth, one from Heilongjiang Province, China (courtesy of Professor Yue Shukui, Northeastern Forestry University, Harbin) and one from Japan (courtesy of Dr. Shimazu Mitsuaki, Forestry and Forest Products Research Institute, Ibaraki Prefecture) (designated here as LdMNPV-H and LdMNPV-J, respectively). The third strain used is the active ingredient in the baculovirus product Disparvirus (designated here as LdMNPV-D), produced by the Canadian Forest Service (CFS) and registered in Canada for gypsy moth control (Health Canada 2009). Disparvirus has the same active ingredient as Gypchek (Cunningham et al. 1991), the product used operationally in the United States for gypsy moth control.

All three virus isolates were amplified under quarantine conditions at PFC twice *in vivo* using laboratory-reared larvae of the Russian strain of gypsy moth (provided courtesy of Vic Mastro and John Tanner, Otis Methods Development Center, USDA/APHIS/PPQ, Massachusetts) at PFC prior to shipping to China. The amplified virus strains were shipped by courier to the senior author as cadavers of Russian strain gypsy moth larvae (AGM-R) on frozen ice packs.

Virus Purification. The virus stocks and solutions were prepared fresh each year of the 3 years of the experiment in Huhhot. Approximately 30-40 days prior to the experiment, 10 g of insect cadavers from each of the virus isolates were macerated separately and the occlusion bodies (OBs) were purified following the procedure described in Ebling et al. (2004). Following purification, the pellets were re-suspended in distilled water, and the OBs quantified using a hemocytometer, (Wigley 1980). After quantification the virus stock solutions were stored at 4°C until 1 day prior to the treatment in the field, at which time the three virus concentrations were prepared.

Virus application. There were four treatments for each of the three geographic isolates of LdMNPV: distilled water for the control, and three virus concentrations

treatments (low, medium and high) for each of the three virus strains (Table 1). The three concentrations (low, medium and high of the three virus strains) that were selected to test in the field in 2004 were based on the results of previous bioassays using the same three strains of virus against AGM-C and AGM-R. The LD_{50} values for LdMNPV-D, LdMNPV-H and LdMNPV-J were 194, 211 and 940 OBs/larva, respectively, against AGM-C (unpublished data) and 1,904, 648 and 1,260 OBs/larva, respectively, against AGM-R (Ebling et al. 2004). For the field trials we selected doses that were the average of the two values and slopes obtained in the previous two laboratory bioassays (given above). There were four treatments (three doses plus one control), and six replicates for each treatment, for a total of 24 cages for each of the three virus isolates tested, for a total of 72 cages for the field test in each year. The experiment was arranged in a randomized block design (Fig. 1). Rooted cuttings growing in rows 1, 3 and 5 were used for treatments, while rooted cuttings in rows 2 and 4 were used as a spray “buffer”. Treatments were assigned randomly to each cluster of caged rooted cutting.

All three geographic strains of LdMNPV were applied to the randomly selected rooted cuttings on the same day in the field in 2004 and 2006 (June 25, and June 24, respectively). In 2005, an unforecasted heavy rainstorm (which deposited 10 mm of rain in 20 minutes, and a total of 15 mm in 26 hours) occurred just after the application of the LdMNPV-D isolate on June 6, forcing the postponement of applications of the remaining two isolates (LdMNPV-H and LdMNPV-J) until the following day (June 7), when the leaves were dry.

The application of the virus strains in 2004 was used as a pilot, or “range-finding”, test to determine the three virus concentrations that would be used in the final field experiment. A separate 600mL hand-held pump sprayer (Model SX-735, Zhe Jiang Shi Xian Sprayer Limited Company, Tai Zhou, Zhe Jiang, China) (Fig. 2b) with a hollow-cone nozzle was used to apply the controls and doses for each of the three virus strains. All the controls (distilled water) were applied first, followed by the three virus strains, in the following order: LdMNPV-D, LdMNPV-H and LdMNPV-J. Treatment with each virus strain started with lowest concentration, and ended with the highest concentration. The control and three virus strains and concentrations were applied in the same order all 3 years.

The steel-frame cages were removed before the application of each treatment. Newspaper-covered cardboard was held around three sides of the rooted cutting in each group of rooted cuttings during treatment to prevent spray drift to adjacent groups of rooted cuttings. About 100 mL (about 25 mL per rooted cutting) of one of the appropriate virus solutions (low, medium and high) was applied to both sides of the leaves of the rooted cuttings in one pit. On average, there were a total of about 100 leaves on the three or four rooted cuttings in each pit. The spray nozzle was held about 20-25 cm from the leaves, and the spray was applied to all sides of the rooted cuttings by the same person each year to minimize variation in application and droplet size. Both the controls and virus solutions were applied until the leaves were dripping. Once the spray had dried on the leaves, the cages were re-positioned over the rooted cuttings. Average spray droplet size was 639.1 ± 30.7 μm, as determined by spraying a microscope slide using the same parameters as those used for the field application, and examining the slide using a dissecting microscope at 10x magnification (Model XTS-20, Beijing Tech Instrument Corporation, Ltd., Beijing, P.R. China).

In 2004 and 2005, 30 and 20 newly molted second instar gypsy moth larvae, respectively, were placed directly onto the rooted poplar cuttings inside each cage after the spray droplets had dried on the leaves. The concentration of

OBs used for the field experiment in 2004 for both the LdMNPV-D and LdMNPV-H gypsy moth virus strains were so high that both the moderate and high concentrations caused 100% mortality of the test larvae. Therefore, the concentrations of 1/10 and 1/100 of the lowest concentration applied in 2004 were used as the low and medium doses in 2005 and 2006, and the lowest concentration in 2004 became the high concentration for both of these strains (LdMNPV-D and LdMNPV-H) in 2005 and 2006 (Table 1). In 2005, an unforecasted rainstorm halted the application of the viruses after the highest concentration of LdMNPV-D had been sprayed onto the foliage, and the remaining two virus strains were not applied until after the foliage had dried the following day. Undoubtedly, an unknown amount of the OBs were washed off the leaves by the rainstorm. As a result of this rainstorm, it was anticipated that larval mortality caused by LdMNPV-D would be reduced. Because of these complications, the whole field experiment was repeated in 2006.

Table 1**. Percent mortality of second-instar larvae of the Chinese strain of Asian gypsy moth caused by three different concentrations of three geographic isolates[a] of LdMNPV in 2005 and 2006, in Huhhot, Autonomous Region of Inner Mongolia, P.R. China.**

Treatment (virus isolate)	Concentrations (OBs/mL)	Larval mortality (%)	
		2005	2006
Control (dH_2O)	0	0	3.4 ± 1.7
Geographic isolates tested			
LdMNPV-D	Low 926	3.4 ± 2.4	*12.8 ± 3.1
	Medium 9,260	12.3 ± 4.0	*31.2 ± 5.6
	High 92,600	21.4 ± 15.8	*64.9 ± 4.4
LdMNPV-H	Low 590	21.3 ± 6.3	14.6 ± 4.5
	Medium 5,900	28.4 ± 9.9	46.5 ± 6.2
	High 59,000	74.8 ± 8.5	75.1 ± 3.2
LdMNPV-J	Low 3,340	50.3 ± 18.0	28.3 ± 6.3
	Medium 33,400	38.5 ± 12.4	43.2 ± 8.7
	High 334,000	90.8 ± 2.6	*71.0 ± 4.1

In 2006, to avoid problems caused by the unexpected rainfall the previous year, two changes were made to the procedure outlined above. First, to reduce or prevent the potential effects of precipitation on the experiment, a 1 m^2 transparent plastic sheet was placed on the top of each of the cages, including the controls, and tied down using nylon rope to protect the treated foliage in the cages from rainfall. These sheets were removed 15 days after the virus application. Second, to facilitate finding all the larvae on the rooted cuttings when checking for mortality, mesh sleeves were used. Once the spray droplets had dried on the leaves, a 25 cm x 30 cm piece of nylon mesh (the same material used to cover the top and sides of the cages), with a nylon zipper running down the short edge (25 cm) so the sleeve could be opened to check larval mortality, was placed around each of three of the treated rooted cuttings in each of the cages. The sleeve was then closed tightly around the rooted cutting at each end using cloth ties sewn to 6-8 cm bands of white muslin (also known as calico) sewn onto each long edge (30 cm side) of the sleeve. The resulting mesh sleeve measured approximately 25 cm long and 9.5 cm in diameter.

Ten newly molted, less than 24-hour-old, second-instar AGM-C larvae, starved for 24 hours, were released into each of the three sleeves in each cage (for a total of 30 larvae per steel frame cage). In all 3 years, larvae in each of the cages were checked every 1 to 3 days and the dead larvae removed. In 2004, the rearings were terminated on day 19 post-inoculation because there was 100% mortality in several cages. In 2005 and 2006, the larvae were reared until pupation began at days 35 and 29, respectively. After the start of pupation, the cages were left in place until all the adults had emerged, mated and the females had laid their eggs and died. The effects of virus doses on egg-laying will be presented in a subsequent paper on the sublethal effects of these virus treatments on AGM-C. Ten percent of all the cadavers were randomly selected from each virus strain, dose and replicate and examined for the presence of OBs under a compound microscope (Model CX21FS1, Olympus Corporation, Tokyo, Japan) at 1000x magnification and oil emersion to calculate percent viral infection based on a random sample.

At the conclusion of the field trials in 2004, and also in 2005, the rooted cuttings were cut back to a height of 10-15 cm above the ground in the spring before growth began to remove the branches with cocoons and egg-masses. All leaf litter and other detritus (including dead AGM-C adults) was also removed and discarded to eliminate any potential sources of inocula the following year. In addition, all the soil within the experimental plot (not just underneath the rooted cuttings) was turned over to bury in the ground any residual virus that may have ended up on the soil surface from virus-killed larvae. These measures were taken to reduce or eliminate any residual active virus on the soil surface or on the stems of the rooted cuttings that could potentially affect the field trials that were conducted the following year. Consequently, only newly grown stems with leaves were used in the experiments in 2005 and 2006. Cages were washed and decontaminated using 1% bleach solution following the field trials in 2004 and 2005, and were dried in the sun (so UV rays could deactivate any virus particles that may have remained on the cages) prior to storing.

Weather data. Temperature, precipitation, relative humidity, wind speed and direction were obtained from a weather station located 8 km from the University Research Area.

Data analysis. AGM-C larval mortalities of caused by the same strains and concentrations in 2005 and 2006 were compared using one-way ANOVA. The LC_{50} and LC_{95} (mean lethal concentration causing 50% and 95% larval mortality, respectively) for each LdMNPV isolates was estimated using POLO- PC (LeOra Software 1994). The LC_{50} and LC_{95} values were examined for significant differences, i.e. no overlap of the 95% confidence limits. Each virus strain had its own control replicate, and all six control replicates were used for comparison with its respective virus strain. The ST_{50} (time required for the virus to cause 50% larval mortality) was determined using ViStat (Hughes and Wood 1990).

Results

Pilot experiment – 2004. The preliminary (“range-finding”) field trial conducted in 2004 indicated the practicality of the proposed method. It also provided data that allowed us to select the virus doses to use in 2005. Results from the field trial in 2004 showed that the moderate and high concentrations of LdMNPV-D (926,000 and 9,260,000 OBs/µL) and LdMNPV-H (590,000 and 5,900,000 OBs/µL) applied to the foliage were too high and caused 100% larval mortality. Consequently, when we repeated the experiment in 2005 and lowered

the doses used. The lowest concentration was retained and we added a 10% and 1% dilutions of the lowest dose of each of these two virus strains for testing in 2005 (Table 1).

There were six replicates for each treatment and 20 larvae per replicate in 2005 (N = 1080 larvae / virus isolate, N = 360 for controls,), 30 larvae per replicate in 2006 (N = 1620 larvae / virus isolate, N = 540 for controls).

[*] Significance at 0.05 level. Significance between the 2 years compared using one-way ANOVA.
[a] LdMNPV-D = active ingredient in Disparvirus, the commercial product registered for gypsy moth control in Canada, LdMNPV-H = virus isolated Chinese strain of Asian bypsy moth north of Harbin, Heilongjiang Province, P.R. China, and LdMNPV-J = virus isolated from Asian gypsy moth in Japan.

Weather. In 2005 an unforecasted rainstorm deposited 10 mm of rain in 20 minutes, and a total of 15 mm in 26 hours occurred immediately following the application of the LdMNPV-D virus strain on June 6. This rainstorm washed an unknown quantity of virus from the three doses of LdMNPV-D off the leaves of the rooted cuttings. Rather than present incomplete data for 2005, we repeated the entire test (applying all three virus strains) in 2006. We compared larval mortality caused by the three virus strains in 2005 with the mortality caused by the same virus strains and concentrations in 2006 to determine the effect of the unforecasted rainstorm on larval mortality for LdMNPV-D. The nearest weather station was located 8 km away, and we used this weather station to approximate the weather conditions among the rooted cuttings in our experimental plot at the time of treatment. Wind speed was not a factor during virus application near ground level among the rooted cuttings. During the 3 years the field trials were conducted, relative humidity among the rooted cuttings at the time of virus application was estimated to be between 30 and 60%, and air temperature ranged between 21 and 27°C. The actual amount of precipitation was measured in the plot. Precipitation only occurred once during the 3-year study, as mentioned above.

Comparison of mortalities of second-instar AGM-C larvae challenged with the same virus concentrations in 2005 and 2006. The application of LdMNPV-D was followed by a heavy rainstorm in 2005 and, as expected, mortality was significantly lower for all three doses [low ($F = 5.81$; df = 1, 10; $P = 0.037$), medium ($F = 6.03$; df = 1, 8; $P = 0.040$) and high ($F = 6.99$; df = 1, 10; $P = 0.025$)] compared to 2006 (Table 1). Mortality caused by low ($F = 0.408$; df = 1, 10; $P = 0.746$), medium ($F = 2.443$; df = 1, 10; $P = 0.149$) and high concentrations ($F = 0.001$; df = 1, 10; $P = 0.978$) of LdMNPV-H virus in 2005 and 2006 did not differ significantly. Similarly, larval mortality caused by the low ($F = 1.332$; df = 1, 10; $P = 0.275$) and medium concentrations ($F = 0.097$; df = 1, 8; $P = 0.762$) of LdMNPV-J in 2005 and 2006 did not differ significantly. However, mortality caused by the highest concentration of LdMNPV-J applied in 2006 was significantly lower than the mortality in 2005 ($F = 16.54$; df = 1, 10; $P = 0.002$).

Dose response in 2006. As previously mentioned, an unforecasted rainstorm occurred immediately following application of the LdMNPV-D virus strain **i**n 2005, reduced larval mortality by 60-75% of that obtained in 2006. The field trial in 2005 demonstrated that the virus doses selected for LdMNPV-H and LdMNPV-J would enable us to determine the LC_{50} and LC_{95} of these two virus strains (Robertson and Preisler 1992). Because the rearings from the 2006 field trials gave the most dependable results and were done in sleeve cages, which allowed for more accurate counting of larval mortality, it was decided that the dose response of AGM-C larvae in the field would be determined using only the 2006

data. Only one larva died in the controls in 2006, all control data was used for calculating the LC_{50} and LC_{95} using POLO-PC (LeOra Software 1994).

As expected, larval mortality increased with increasing virus dose (Table 1). LdMNPV-H was the most pathogenic of the isolates tested against larvae of the AGM-C in the field, having the lowest LC_{50} and LC_{95} (6.5 and 742 OBs/µL, respectively). LdMNPV-H was marginally more pathogenic than LdMNPV-D at the LC_{50} level (requiring fewer OBs), but not at the LC_{95} level (where there was overlap of the 95% confidence limits) (Table 2). The upper 95% confidence limit for LC_{50} of LdMNPV-H and lower 95% confidence limit for LdMNPV-D were separated by only 8 OBs.

The LC_{50} (14.7 OBs/µL) and LC_{95} (11,542 OBs/µl) of LdMNPV-J was not significantly different from the respective corresponding values of the other two isolates (the 95% confidence limits overlapped). LdMNPV-D had a LC_{50} (31.5/µL OBs) and LC_{95} (4,093 OBs /µL), while LdMNPV-J had not only the highest LC_{95}, but also the widest 95% confidence limit of the three isolates. We therefore considered LdMNPV-D to be biologically more pathogenic than LdMNPV-J because of its lower LC_{95} (4,093 OBs/µL) and much narrower 95% confidence limits, even if the LC_{50}s and LC_{95}s for the two isolates were not significantly different.

The slope of the regression line obtained in Probit analysis has generally been interpreted as a measure of variability in host susceptibility to the virus; with less variation among host larvae producing steeper slopes (Hughes et al. 1984). Insects completely homogenous in susceptibility would yield a slope of about 2 (Burges and Thomson 1971), with less variability between hosts producing steeper slopes. Slopes less than 2 are due to insect variability rather than problems in methodology (Ebling et al. 2004). All of the slopes presented in Table 2 are less than 2, supporting the suitability of methodology used to conduct this field experiment with the three virus strains. The steepness of the slopes of the dose-response regression lines for the AGM-C larvae to the three viral isolates tested indicated that AGM-C larvae had a more homogenous susceptibility to LdMNPV-H and LdMNPV-D than that to LdMNPV-J.

Time-response. The speed with which a virus causes mortality of the host is an important measure of its effectiveness as a biopesticide when foliage protection of the host tree is desired. Time response was only calculated on the 2006 data, because these results were complete for all three virus strains and most accurate, due to the use of sleeve cages. Results of the time-response (amount of time required to kill 50% of the larvae, ST_{50}) for the field applications of the three virus strains against AGM-C larvae are presented in Table 2. There were no significant differences in the time-response among the three replicates within each virus strain; therefore data sets for each virus strain were pooled for analysis with ViStat. The time-response data for the larval mortality caused by LdMNPV-D fits the model, because the observed χ^2 values are significantly lower than predicted values ($\chi^2 = 9.7$, df = 7, P = 0.2062). However, this was not the case for either LdMNPV-H ($\chi^2 = 28.1$, df = 8, P = 0.0004) or LdMNPV-J ($\chi^2 = 60.6$, df = 9, P < 0.0001).

The time-response data (ST_{50}) indicates that LdMNPV-J had a significantly faster time response (ST_{50} 11.1 d) than the other two isolates tested (the 95% confidence limits did not overlap those of the other two strains). However, the dose causing this faster “kill” response was much higher for LdMNPV-J than for the other two strains (Tables 2 and 3). LdMNPV-H was also significantly more virulent (ST_{50} 13.5 d) than LdMNPV-D (ST_{50} 14.7d) (Table 2).

Table 2. **Dose- and time-response of second-instar Chinese strain of Asian gypsy moth larvae to three geographic isolates of LdMNPV in field tests at Huhhot, Inner Mongolia, P.R. China, in 2006.**

Strains	Dose-Response (OBs/µL)					Time-Response (day)		
	N	Slope±SE	LC_{50} [a]	LC_{95} [a]	χ^2/df	ST_{50} [b]	Slope±SE	χ^2/df
LdMNPV-D	1,620	0.78 ± 0.10	31.5 (20.2 - 54.6)	4,093 (1,261 – 26,781)	8.38 / 10	14.7 (14.2 - 15.4)	10.31 ± 1.05	9.7 / 7
LdMNPV-H	1,620	0.80 ± 0.10	6.5 (4.9 - 12.4)	742 (249 – 4,406)	8.17 / 10	13.5 (13.0 - 14.1)	9.06 ± 0.77	28.1 / 8
LdMNPV-J	1,620	0.57 ± 0.09	14.7 (7.3 - 25.6)	11,542 (2,682 – 162,853)	7.8 / 10	11.1 (10.7 - 11.5)	8.61 ± 0.67	60.6 / 9

[a] Calculated using probit analysis (LeOra Software, 1994) statistical package; numbers in parentheses indicate 95% confidence limits.

[b] Determined using pooled replicates having less than 50% mortality; calculated using ViStat (Hughes and Wood, 1990) statistical package; numbers in parentheses indicate 95% confidence limits calculated as being double the SE.

Discussion

As expected, mortality generally increased with increasing virus dose for all three virus strains, with the exception of the medium dose of LdMNPV-J in 2005 (Table 1). We have no explanation for this anomaly, apart from this being the result of an unexplained experimental error. There were no real significant differences in pathogenicity of the same doses of LdMNPV-H and LdMNPV-J isolates tested against AGM-C in 2005 and 2006, with the exception of the highest dose for LdMNPV-J. Again, we think this is due either to experimental error or to the application of the treatment itself, since both exceptions occur with this virus strain.

Mortality values for the three doses of LdMNPV-D in 2005 were about one-third of that caused by the same three doses in 2006 (Table 1). This significantly reduced mortality was the result of the unexpected heavy precipitation (10 mm of rain in 1 hour, and a total of 15 mm in 26 hours) immediately after the LdMNPV-D strain was applied. This "unplanned experiment" showed that heavy precipitation immediately after the virus application, before the virus has a chance to dry onto the leaves, can reduce larval mortality by about 60-75%. D'Amico and Elkinton (1995), using artificial rainfall (equivalent to 2.4 cm precipitation in a 5 minute period) and natural rainfall (1.4 cm precipitation over an unspecified period of time), has the effect of washing viral OBs from leaves and decreasing mortality of gypsy moth larvae consuming the leaves in the upper crown of the tree. Unlike D'Amico and Elkinton (1995), we found that the rainfall on leaves did not appear to cause increased virus-caused larval mortality on leaves located on the lower crown of the tree, nor did the rainfall appear to move virus from branch to branch. The differences between our results and those of D'Amico and Elkinton (1995) are likely due to our use of rooted cuttings (less than 1 m tall), rather than full sized trees.

The results of the field test in 2006 suggest some interesting trends. LdMNPV-H had a significantly lower LC_{50} than LdMNPV-D, but was not significantly pathogenic than LdMNPV-J. There were no significant differences of LC_{95} values among the three isolates (LdMNPV-H, LdMNPV-H and LdMNPV-J). Overall, LdMNPV-H appeared to be the most pathogenic strain of the three geographic isolates of LdMNPV tested in the field against second-instar larvae of the local AGM-C, indicated by having the lowest LC_{50} (6.5 OBs /µL) and LC_{95} (742 OBs/µL), and having the narrowest 95% confidence limits of the three virus stains tested (Table 2). However, this significantly higher pathogenicity may be more apparent than real, because the difference is based on a difference of only 8 OBs. These results suggest that, based on pathogenicity in the field trial, the LdMNPV-H strain would be superior for controlling the AGM-C. On the other hand, the slight difference in pathogenicity between LdMNPV-H and the currently registered strain (LdMNPV-D), in our opinion, would not justify the added expense that would be required to register LdMNPV-H.

In a specifically designed study with another insect from the same family, Douglas-fir tussock moth (*Orgyia pseudotsugata* (McDunnough)), we compared the LC_{50} values obtained using the diet surface contamination technique obtained with LD_{50} values using the diet plug method. Results showed that the LC_{50} of larvae challenged using diet surface contamination was 1.4 to 2.0 times higher than the LD_{50} of larvae challenged using diet plugs (unpublished data). Based on the results of these bioassays, we expected similar results when we compared the LC values from the field trial presented in this paper with the LD values in our laboratory bioassay (unpublished data).

The LD values, from the laboratory bioassays, were obtained by feeding a known quantity of virus to the larvae using diet plugs (the entire dose is consumed). The LC values, from the field trials, were obtained by applying virus to leaf surfaces and allowing larvae to feed on the treated foliage. In the latter case, we only had control over how much virus (quantity of inoculum) was applied to each leaf, and no control on how much foliage (and virus) was consumed by each individual larva. Because of the uncertainties of the latter method, the diet plug (LD) method is more accurate than diet surface or leaf surface contamination methods. It is difficult to compare the results of field tests (LC values with bioassay results (LD values), and we are not aware of any published work that makes this comparison.

In the field trial reported here, we found the biological activities of all three LdMNPV isolates generated much lower LC_{50}s and LC_{95}s in the field against AGM-C compared with the LD_{50} and LD_{95} observed in the laboratory bioassays against second-instar AGM-C larvae. This was contrary to expectations.

In both laboratory and field experiments, LdMNPV-H was the most pathogenic of the three virus isolates tested against AGM-C. In the field, LdMNPV-H was more pathogenic than LdMNPV-D against AGM-C, but in laboratory bioassays both viruses had similar pathogenicity against AGM-C (LD_{50} of 211 OBs and LD_{95} of 1,414 /µL for LdMNPV-H compared with LD_{50} of 194 OBs and LD_{95} of 1,705 OBs/µL for LdMNPV-D) (unpublished data). Also, while the laboratory bioassay indicated that LdMNPV-J was the least pathogenic against AGM-C, in the field trial the results were somewhat less conclusive. The LC_{50} for LdMNPV-J in the field (14.7 OBs/µL) was in between the LC_{50} values for the other two isolates, while the LC_{95} (11,542 OBs/µL) was almost identical to the LD_{95} (11,542 OBs / larva) in the laboratory bioassay.

All three virus strains took longer to cause mortality in the field compared to the laboratory bioassays. This was expected. In the field the ST_{50} for LdMNPV-D and LdMNPV-H increased by about 5 days, and for LdMNPV-J the ST_{50} increased by about 2.5 days. In the field, LdMNPV-J had the highest virulence, having a significantly lower ST_{50} (11.1 d) than the other two strains. However, this occurred at a much higher dose than for the other two virus strains. Of the remaining two strains, LdMNPV-H was more virulent than LdMNPV-D, but the difference in virulences was only barely significant (there was a 0.1 d separation in the 95% confidence limits). These differences in the ST_{50}s were expected, because larvae in the laboratory bioassay were reared at constant temperature of 25°C with a constant supply of food, while the larvae in the field experiment were subjected to fluctuating temperatures and needed to move in search of food. Virus replication is slower at lower temperatures, but the cumulative virus mortality at lower temperatures is similar of that achieved at optimal temperature (Boucias et al. 1980). During this field trial, the ambient temperature ranged from 13°C to 32°C, which likely slowed the rate of virus replication compared to what it was at a constant temperature of 25°C during the laboratory bioassay. It is also interesting to note that LdMNPV-J, the least pathogenic of the three strains tested, was the most virulent (it had the lowest ST_{50}), and not LdMNPV-H, which was the most pathogenic strain in both the laboratory bioassay and field trial. Larval mortality by LdMNPV-H and LdMNPV-D started later in the AGM-C on the rooted cuttings, but ended up being higher. It appears that LdMNPV-J is faster acting, but the dose required to achieve this shorter ST_{50} is about 16 times and 3 times higher than for LdMNPV-H and LdMNPV-H, respectively (Table 2).

The low pathogenicity exhibited by LdMNPV-J against the EGM and AGM-R (Ebling et al. 2004) and the native AGM-C (unpublished data) may be related to the subspecies of gypsy moth from which the virus was initially isolated, and/or phase of the outbreak cycle. We have no information on the phase of the outbreak where LdMNPV-H and LdMNPV-J were collected. Amplifying the three virus strains twice in AGM-R should, theoretically, have reduced or eliminated any major differences caused by this factor (outbreak phase). According to Pogue and Schaefer (2007), neither EGM nor AGM were reported to occur in Japan, and of the species of gypsy moth in the subgenus *Porthetria* reported in Japan, only *L.d. japonica* is recorded from the Ibaraki Prefecture where the virus-infected larvae were likely collected. It would be interesting to test the same three geographic variants of LdMNPV used in this study against *L.d. japonica* to see if LdMNPV-J exhibits greater virulence against the Japanese subspecies of gypsy moth. Due to international trade, Japan is one potential source of gypsy moth introduction from Asia.

The lower LC_{50}s (increased potency) can be explained, we think, by several factors. First, the virus was applied to the foliage until the leaves were dripping. Since the virus was applied to leaves (which have a comparatively large surface area), rather than diet (which has a smaller surface area) being offered as a food source to the larvae, the larvae were probably exposed to higher levels of virus (inoculum) in the field trial (feeding on contaminated leaves) than they were during the laboratory bioassay (feeding on a 4.4±0.1 mg inoculated diet plugs or contaminated diet). Second, larvae feeding on foliage that was sprayed until dripping would have more time and opportunity to ingest a lethal dose of the virus than the 24-48 hours the larvae had to feed on inoculated material in the bioassay. The mesh sleeves used in the cages may also have offered the virus on the leaves some partial protection from deactivation by UV light. Even during diet surface contamination experiments, second instar larvae were never able to consume more than one-quarter of the contaminated diet, because the diet had to be changed once a week to prevent it from desiccating or becoming moldy. Third, there are the additive and synergistic effects on insect virus activity that allelochemicals, substances that are produced by the host plant as defense mechanisms.

The susceptibility of gypsy moth, *Lymantria dispar* (L.), to LdNPV has been shown to be affected by changes in host plants (Keating and Yendol 1987, Keating et al. 1988). Gypsy moth larvae consuming virus- treated foliage of aspen (*Populus* spp.) had higher mortality than did larvae consuming virus-treated oak (*Quercus* spp.) foliage. Larvae on oak were less susceptible to infection while those on aspen were more susceptible. In a later study, Keating et al. (1990) reported that diet pH may also affect virus activity by changing midgut pH of the defoliator and altering the rate and location of virion release in the midgut lumen. The ability of gypsy moth virus and other baculoviruses to cause fatal infection was reported to be reduced significantly when larvae consume foliage high in polyphenols (Keating et al. 1988, 1989; Hunter and Schultz 1993). The increasing levels of tannin in oak leaves as the leaves age have been reported to negatively affect infection by LdNPV in the gypsy moth (Barbosa and Krischik 1987, Schultz 1989, Keating et al. 1989, Hunter and Schultz 1993).

Phenolic glycosides are plant defense chemicals known to enhance the insecticidal activities of *Bacillus thuringiensis* (Arteel and Lindroth 1992) and entomopathogenic viruses (Keating and Yendol 1987, Cook et al. 2003. Four phenolic glycosides, salicin, salicortin, tremulacin and tremuloidin, have been

identified in *Populus tremuloides* Michaux, of which one, salicortin, has been described as occurring in all members of the genus *Populus* (Pearl and Darling 1971). Cook et al. (2003) indicate that the phenolic glycoside salacin in *P. tremuloides* enhances the virulence of Gypchek against EGM, reducing the LD_{50} by approximately 25%. This is supported by Keating and Yendol (1987), who report that the host tree alters the efficacy of the dose the EGM larvae receive, reducing the LD_{50} by as much as 70% (LD_{50} for EGM on red oak was 33,651 PIBs/larva compared with LD_{50} of 10,165 for EGM on aspen). Although the quantity of phenolic glycosides in the leaves of *P. cathyana* has not been studied, it is known that the phenol content of *P. cathayana* phloem is higher than that of at least four other species of poplar in China *(P. alba var. pyramidalis*, *P. beiijingensis* W.Y. Hsu, *P. hopeiensis* Hu et Chow, and *P. nigra var. thevestina*) (Wang et al. 1987). This may be another reason for the low LC_{50} of second instar AGM-C larvae feeding on *P. cathayana* in this experiment.

In conclusion, LdMNPV-H seems to be the most effective strain tested against AGM-C, but this virus strain has not been registered for use in North America. Currently, the bacterium *Bacillus thuringiensis* subsp. *kurstaki* (Btk) is used in gypsy moth eradication programs in western North America, partly because the product is available commercially, is effective against low density populations of gypsy moth, and can be applied several times in a season. Since Btk is available and is a viable control agent, there is no need to develop LdMNPV-H as a biopesticide. In addition, there are currently two registered virus products (Gypchek in the United States, Disparvirus in Canada) which contain LdMNPV-D as the active ingredient, and could be used to control AGM-C should it become established and Btk not be available as a control tool. Presently, several applications of Btk in one season offers the best chance for eradicating gypsy moth infestations over small to mid-sized areas, but it might be worth repeating the bioassay experiment against the *L.d. japonica*, the gypsy moth with the widest distribution in Japan.

Acknowledgements

We wish to thank Les Safranyik for his advice on the randomized plot design used for this experiment, and Peter Ebling for his assistance with using and interpreting the results of the statistical analysis from both PoloPC and ViStat. We also wish to thank the following students from the Inner Mongolia Forestry College, for checking larval mortality, and without whose help this experiment could not have been conducted: Wang yin, Yang Gao Peng, Lu Hui, Li Dong Wei, and Wang Xiao Qin. Funding for the field experiment and publication was provided by the Canadian Forest Service. Student salaries were paid in part by the National Science and Technology Fund (P.R. China), awarded to the senior author (L.D.).

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COMPARISON OF THE ACTIVITIES OF THREE LDMNPV ISOLATES IN THE LABORATORY AGAINST THE CHINESE STRAIN OF ASIAN GYPSY MOTH

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摘要：三株来自中国、日本和北美的舞毒蛾核型多角体病毒分离株在内蒙古进行了生物测定。2004年，以2龄舞毒蛾幼虫为试虫，通过对病毒的剂量、死亡时间比较，应用校正死亡率方法检测了各分离株的致病性和毒力。结果表明，LdMNPV-H 和 LdMNPV-D 毒株的剂量毒力区别不大，LdMNPV-H毒株的 LD_{50} 和 LD_{95} 分别为211和1414 OBs/头，LdMNPV-D毒株 LD_{50} (194 OBs/头)稍低，而LD_{95} (1705 OBs /头)稍高。LdMNPV-J毒株致病力最低，其中LD_{50} 为940 OBs/头，LD_{95} 为 11457 OBs/头。总体上，LdMNPV-H 和 LdMNPV-D 毒株对中国舞毒蛾亚种2龄幼虫的致病力近似，但致病力均高于LdMNPV-J。而3株病毒对中国舞毒蛾亚种的致病时间没有显著差异。

关键词：杆状病毒；分离株; 致病性；毒力; 舞毒蛾

СРАВНИТЕЛЬНАЯ АКТИВНОСТЬ ТРЁХ ИЗОЛЯТОВ ВИРУСА ЯДЕРНОГО ПОЛИЭДРОЗА НЕПАРНОГО ШЕЛКОПРЯДА В ЛАБОРАТОРИИ ПРОТИВ АЗИАТСКОЙ ФОРМЫ НЕПАРНОГО ШЕЛКОПРЯДА

Три изолята вируса ядерного полиэдроза гусениц непарного шелкопряда *Lymantria dispar* из Китая, Японии и зарегистрированный изолят из Северной Америки были испытаны в 2004 г. в лабораторных условиях во Внутренней Монголии (Китай) с использованием для выкармливания модифицированной питательной среды. Для оценки эффективости изучаемых штаммов использовали дозу препарата (LD_{50}) и время достижения 50%-ной смертности гусениц. В опытах использовали гусениц второго возраста азиатской формы непарного шелкопряда из Китая.

Полученные результаты показали, что штаммы вирусов из Китая и Северной Америки принципиально не отличались по величине LD_{50} , тогда как японский штамм имел более низкие показатли смертности. Время достижения 50%-ной смертности принципиаильно не отличалось для всех трёх испытанных штаммов.

Ключевые слова: бакуловирус, изоляты, патогенность, вирулентость, азиатский непарный шелкопряд.

ABSTRACT: Three isolates of gypsy moth [*Lymantria dispar* (L.)] nucleopolyhedrovirus (LdMNPV), from China (LdMNPV-H), Japan (LdMNPV-J) and the registered strain from North America (LdMNPV-D), were bioassayed in the Inner Mongolia Autonomous Region, People's Republic of China, in 2004, using a modified version of the diet plug method, to determine their pathogenicity and

virulence based on the dose- and time-response of second-instar larvae of the Chinese strain of the Asian gypsy moth. Results showed that LdMNPV-H and LdMNPV-D did not differ significantly in dose-response. The LD_{50} and LD_{95} for LdMNPV-H were 211 and 1414 OBs $larva^{-1}$, respectively, while LdMNPV-D had a somewhat lower LD_{50} (194 OBs $larva^{-1}$) and a higher LD_{95} (1705 OBs $larva^{-1}$). LdMNPV-J was the least pathogenic, with a LD_{50} of 940 OBs $larva^{-1}$ and LD_{95} of 11 457 OBs $larva^{-1}$. Overall, LdMNPV-H and LdMNPV-D had similar pathogenicity against second-instar larvae of the Chinese strain of the Asian gypsy moth, and were considerably more pathogenic than LdMNPV-J. Time-responses were not significantly different among the three virus strains.

Key words: baculovirus; isolates; pathogenicity, virulence; *Lymantria dispar*

Introduction The gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), is a polyphagous defoliator of Eurasian origin known to feed on over 300 species of trees and shrubs. Oaks (*Quercus* spp.) are the favoured hosts [1]. Two strains of gypsy moth are commonly recognized in the literature, the European strain, originating from Europe (including European Russia) and North Africa, and the Asian strain from central and eastern Asia [2].

The European strain of the gypsy moth (EGM) was brought to North America in 1869 in an attempt to improve silk production. However, a few specimens accidentally escaped during a storm and became established in the north-eastern United States (US) [3]. The spread of the EGM in the US is well-documented, and this strain now occurs throughout the north-eastern US [4-5]. EGM has also spread into Canada and is now found from Nova Scotia to mid-western Ontario, causing light to severe defoliation over large areas in Ontario and Quebec [6].

Several methods and various control agents have been used in the US, first to control it, then to slow down the spread of the gypsy moth [4, 7, 8]. During its periodic outbreaks, widespread defoliation (an average of 1.2 million ha annually) occurs when control measures are not applied [9]. It is estimated that approximately $11 million (US) is spent annually in the US on gypsy moth control [10].

Recently, a revised nomenclature for gypsy moths was proposed based on wing pattern and the ability of the female to fly [2]. According to the proposed nomenclature, the subgenus *Porthetria* includes several closely related *Lymantria* spp. The common name “gypsy moth”, *L. dispar dispar* (L.), now denotes only the winged but flightless EGM and the nearly wingless gypsy moth occurring in India, while the term “Asian gypsy moth”, *L. dispar asiatica* Vnukovskij (AGM), refers to the strain that has winged females capable of flight. *L.d. asiatica* is found in Russia east of the Ural Mountains, the northern two-thirds of China, and Korea. In addition, four species of gypsy moth in the subgenus *Porthetria*, *L. albescens* Hori and Umeno*, L. postalba* Inoue, *L. umbrosa* (Butler), and *L. xylina* Swinhoe, and one subspecies, *L.d. japonica* (Motschulsky), occur in Japan. All of the females of gypsy moths occurring in the Japanese archipelago and nearby islands have functional wings. Of these, the Japanese gypsy moth, *L.d. japonica*, has the widest distribution throughout the Japanese mainland.

As a result of increased global commerce, repeated accidental introductions of the exotic Asian strain have occurred in North America, but so far all of these introductions (mostly from Siberia and possibly also from China) have been successfully eradicated [8, 11]. The AGM poses a more serious threat than the EGM for three reasons: the adult females of the Asian subspecies can fly, unlike the winged but flightless European females; the larvae of the Asian subspecies also

feed on conifers [12]; and the Asian subspecies is one of the most widespread defoliators of deciduous and larch forests in China, where it periodically reaches outbreak levels [13]. In addition, AGM and EGM can hybridize, and many of the resulting females can also fly [14, 15]. The ability of both hybrid and purebred females to fly will likely allow the AGM to spread throughout North America much more rapidly than the European strain has so far, and cause more devastating economic losses [16]. Thus, the introduction of the Asian subspecies presents a far greater threat to the coniferous forests of Canada and the US than that posed by its European relative.

The gypsy moth has several naturally occurring infectious diseases, one of which, a type of baculovirus known as a multi-capsid nucleopolyhedrovirus (LdMNPV). It is a major factor controlling *L. dispar* populations in North America [17]. LdMNPV has been observed to reach epizootic proportions as larval densities increase [18]. During the latter half of the last century, research on NPV in several countries culminated in the commercial development of baculovirus insecticides for control of gypsy moth in the US, Canada and the former Soviet Union.

With the increase in international trade, it is possible that the AGM will likely become established. Russia, China, South Korea and Japan are four likely sources for these introductions because of increasing trade between Asia and North America. To prepare for the eventual potential establishment of the AGM in North America, three geographic isolates of LdMNPV were tested against laboratory colonies of the EGM and the AGM strain from Siberia, Russia (AGM-R) [16]. Restriction enzyme digestion profiles indicated that these three virus strains were similar yet distinct, and the isolates can be easily distinguished [16]. One of the three virus strains was isolated from the AGM from Heilongjiang (LdMNPV-H) Province, P.R. China. The second field-collected strain tested (LdMNPV-J) was probably obtained from *L.d. japonica*, the most widely distributed gypsy moth in Japan. The third strain tested was the LdMNPV used in Disparvirus® (LdMNPV-D), a baculovirus product registered for gypsy moth control in Canada [19]. This is the same strain as the one registered in the US under the trade name Gypchek [20].

Ideally, it would have been best to simultaneously bioassay all three virus strains with the three insect strains (EGM, AGM-R and AGM-C) at the same time in the same laboratory. However, quarantine requirements, as well as lack of finances and manpower, forced us to do this comparison in two stages. In the first stage, the three virus strains (D, H and J) were tested in Canada in 2001 and 2002 against EGM and AGM-R, respectively. In the second stage, the same three virus strains were tested against the AGM from China using the same laboratory rearing conditions. This second set of bioassays was done in the Inner Mongolia Autonomous Region, People's Republic of China, where the insect is native.

In this paper, we report on the results of the second stage of the investigation. We compare the pathogenicity and virulence of the same three geographic isolates of LdMNPV against the Chinese strain of the Asian gypsy moth (AGM-C) in the laboratory in Huhhot, Inner Mongolia Autonomous Region, People's Republic of China in 2004.

Materials and methodology

Test Larvae

Egg masses of AGM were collected in March, 2004, from the main host tree, Prince Rupprecht's larch, *Larix principis-rupprechtii* Mayr, in He-Lin County, Inner Mongolia Autonomous Region (150 km south of Huhhot, P.R. China) and stored at 5°C for 2 months.

Starting May 1, eggs were gently separated from the egg masses by hand. Surface decontamination was performed by wrapping the eggs (approximately 500 eggs per package) in a single layer of cheesecloth and immersing them in a 0.05% sodium hypochlorite solution for 30 seconds, followed by rinsing in five distilled water rinses for 1 min each. The eggs were then placed on filter paper to air-dry at room temperature. Approximately 500 eggs were placed into each of 30 fluted containers [6 oz. (177 ml) OZ6-XE6] and covered with cardboard lids (DS306) (Sweetheart Cup Co., Owing Mills, Maryland). The eggs were reared at 25ºC, 60% RH, and 16L:8D photoperiod, i.e. the same conditions used in the first stage in Canada [16]. After hatching, 100 larvae were transferred to each of 150 cups (described above) containing a modified artificial gypsy moth diet [21, 22] and reared at 25ºC, 60% RH, and 16L:8D photoperiod. Newly moulted (less than 24h old) second-instar larvae were starved for 18-24 h before being inoculated with a virus-contaminated diet plug.

Virus Inoculum

The activity of three geographic isolates of gypsy moth virus was tested in bioassays in the laboratory. One isolate, LdMNPV-D (Disparvirus®), was produced and supplied by Dr. John C. Cunningham, Natural Resources Canada, Canadian Forest Service (CFS), Great Lakes Forestry Centre, Sault St. Marie, Ontario, Canada, from the registered virus product, Disparvirus® (Pest Management Regulatory Agency, Health Canada, Registration number 24869). The other two virus isolates were originally collected from field populations of gypsy moths. One of these two isolates, LdMNPV-H, was collected in Heilongjiang Province of China near Harbin (courtesy of Professor Yue Shukui, Northeastern Forestry University, Harbin). The other isolate, LdMNPV-J, was collected in Japan (Ibaraki Prefecture, Honshu Island; courtesy of Dr. Shimazu Mitsuaki, Forestry and Forest Products Research Institute) [16].

All three LdMNPV strains were amplified twice *in vivo* and mass-produced under quarantine conditions at the Pacific Forestry Center (PFC), Natural Resources Canada, Canadian Forest Service, using freshly moulted fourth instar AGM-R larvae. The laboratory colony of AGM-R used for the amplification and mass-production were obtained from the Otis Methods Development Center, USDA, APHIS, PPQ, Massachusetts (courtesy of Dr. Vic Mastro and Mr. John Tanner). The mass-produced virus was stored for 2 years in insect cadavers in 50 ml centrifuge tubes at -20ºC. The insect cadavers were shipped to China for processing in early 2003, among freezer packs in a Styrofoam cooler. Virus occlusion bodies (OBs) were purified following the procedure described previously [16]. The stock suspension of OBs for each LdMNPV strain was prepared 1 month before the bioassays were conducted, and stored at 4°C until use. OBs were quantified using a haemocytometer.

Dose-response

Freshly moulted second-instar AGM-C (*L.d. asiatica*) larvae were starved for 24 h and then inoculated with 1 µl of virus suspension one of the three strains of LdMNPV using a modified version of the contaminated diet-plug method [23, 24] to determine the dose and time-response to each LdMNPV isolate. Control larvae were fed a diet plug inoculated with distilled water (dH_2O). Virus inoculations targeted 5, 30, 50, 80, 85, 90 and 95% mortality (determined by preliminary bioassays), thereby giving the narrowest confidence limits for both the LD_{50} and LD_{95} [25].

During the preliminary bioassays of all three LdMNPV strains, a concentration close to the LD_{50} for LdMNPV-D was determined. However, the results of the Probit analysis for the other two virus strains either did not fit the

Probit curve or the replicates differed substantially from each other, so an analysis of the pooled data could not be performed and no doses approximating the LD_{50}s could be determined for either LdMNPV-H or LdMNPV-J in 2003. Because of the possibility that the outbreak would collapse (depriving us of a source of larvae for the bioassay) and monetary constraints, it was decided not to repeat this preliminary bioassay, but instead to add two extra doses to improve the chances of calculating the LD_{50} and LD_{95} dose for LdMNPV-H or LdMNPV-J. Therefore, for the full bioassay, for LdMNPV-D, five concentrations were used to determine the LD_{50}, while for LdMNPV-H and LdMNPV-J two extra doses were used (total of seven concentrations) to determine the LD_{50} and LD_{95} in 2004.

The starved larvae were reared in darkness at 25 ± 1 ºC, 60% RH and allowed to feed for 24 h on the treated diet. Larvae from laboratory colonies are used to eating this artificial (substitute) diet and readily accept this diet. After 24 h, larvae that had consumed the entire plugs were transferred individually to Solo PL1 plastic souffles (29.6 ml cups, Solo Cup Company, Urbana, IL), containing untreated fresh diet and capped with Solo PL1 paper lids, and reared at 25 ± 1ºC, 60% RH, and 16L:8D photoperiod. Larvae that did not consume the entire plug were discarded. Bioassay of each viral strain dose and a corresponding experimental control group was replicated three times with 72 larvae per replicate, for a total of 216 larvae per concentration, for each viral isolate. Food was changed weekly and the larvae were monitored daily for mortality. Moribund larvae were considered as dead and gently prodded with a probe. Mortality caused by virus was diagnosed from typical virus disease characteristics (soft, flaccid body) and confirmed by microscopic examination. Larval mortality was analyzed using Probit analysis [26] to estimate the LD_{50}, LD_{95}, and 95% confidence limits.

Time-response

Second-instar AGM-C larvae were treated using virus-inoculated diet plugs and then reared on non-contaminated diet. The virus doses were standardized to produce similar mortality rates in order to facilitate comparisons among the three virus strains. Equally effective doses causing less than 50% mortality were chosen, because the ST_{50}s generated from these data are relatively insensitive to dose differences [27]. Data obtained from the dose-response bioassays for each viral isolate were analyzed to determine ST_{50} using ViStat [28].

Results

Dose-response

There were no significant differences in dose-response (LD_{50} and LD_{95}) among the three replicates for each of the three LdMNPV strains, as determined by overlapping 95% confidence intervals [26]. Therefore, the three replicates for each virus strain were pooled and the combined data were used for the Probit analysis of the viral strains' dose-response. Mortality in the control groups was very low (2.8%). Natural response was estimated [26] for the regression of data sets in which control mortality occurred. The experiment was terminated 21 d post-inoculation, and no pupation occurred within this observation period.

The dose-response bioassays of larvae of AGM-C showed that LdMNPV-H and LdMNPV-D had very similar activity against the second-instar AGM-C larvae. The median lethal doses (LD_{50}) for LdMNPV-D and LdMNPV-H were 194 and 211 OBs $larva^{-1}$, respectively, and were not significantly different (i.e. there was overlap of 95% confidence limits of LD_{50}). Similarly, LD_{95}s were not significantly different at 1705 and 1414 OBs $larva^{-1}$, respectively. LdMNPV-J was significantly less pathogenic against the AGM-C (the 95% confidence limits did not overlap), having the highest LD_{50} and LD_{95} (940 and 11 457 OBs $larva^{-1}$, respectively) (Table 1).

Table 1. **Response of second-instar larvae of the Chinese strain of *Lymantria dispar asiatica* to three geographic isolates (LdMNPV-D, LdMNPV-H, and LdMNPV-J) of nucleopolyhedrovirus in laboratory bioassay, Huhhot, P.R. China, 2004.**

Isolate	Dose response					Time response		
	LD_{50}[a] (OBs $larva^{-1}$)	LD_{95}[a] (OBs $larva^{-1}$)	Slope±SE	χ^2/df[b]		ST_{50}[c] (day)	Slope±SE	χ^2/df[d]
LdMNPV-D	194 (157 - 235)	1705 (1254 – 2541)	1.74 ± 0.14	12.72 / 13		8.85 (8.45 - 9.25)	10.31 ± 1.17	9.54 / 9
LdMNPV-H	211 (175 - 255)	1414 (1066 – 2011)	1.99 ± 0.14	6.25 / 11		8.33 (8.02 - 8.65)	10.89 ± 1.10	28.36 / 11
LdMNPV-J	940 (748 – 1167)	11 457 (7768 – 19 366)	1.51 ± 0.13	6.17 / 10		8.61 (8.19 - 9.03)	8.18 ± 0.80	23.88 / 12

[a] calculated using Polo PC [26]; numbers in parentheses indicate 95% confidence limits.

[b] the degrees of freedom are less than 14 and differ among the analyses because data points considered “outliers” (dose responses that were abnormally high or low) were excluded from the final determination of the LDs for each virus.

[c] determined using pooled replicates causing less than 50% mortality; calculated using ViStat [28]; numbers in parentheses indicate 95% confidence limits and were calculated as twice the standard error of the ST_{50}.

[d] degrees of freedom differ because of the number of days that virus-induced mortality occurred varied among the three LdMNPV strains.

The slope of the regression line in Probit analysis has generally been interpreted as a measure of variability in host susceptibility to the virus [29], with less variation among hosts producing steeper slopes. Slopes of the dose responses for the three isolates (Table 1) indicate that AGM-C is equally susceptible to LdMNPV-H and LdMNPV-D and least susceptible to the infection by LdMNPV-J. Both LdMNPV-H and LdMNPV-D isolates caused higher mortality of AGM-C larvae infected in the second instar, than LdMNPV-J isolate. Regression lines for the dose- response of AGM-C larvae to the three virus strains were parallel, and the ratio of relative potency of LdMNPV-H and LdMNPV-D to LdMNPV-J was estimated [25, 26] to be 4.5 and 4.8 fold, respectively.

Time-response

Virulence, best determined by the time-response, is the length of time it takes for the virus to cause mortality and is an important measure of the effectiveness of the virus as a biopesticide [27]. The virulence of the three LdMNPV strains to second-instar AGM-C larvae was compared in the term of median time response, ST_{50} (time required to kill 50% of the larvae), at the equally effective dose. There were no significant differences in the time-response (ST_{50}) among the replicates (receiving doses causing somewhat less than 50% mortality) within each of the three virus strains bioassayed. Therefore, data sets were pooled for each of the virus strains prior to analysis with ViStat [28].

The time-response data indicated that all three strains of the LdMNPV have similar ST_{50}s, as determined by the overlapping 95% confidence limits. LdMNPV-D, LdMNPV-H and LdMNPV-J had ST_{50}s of 8.9, 8.3 and 8.6 d, respectively. However, for two of the three virus strains tested (LdMNPV-H and LdMNPV-J), the observed χ^2 values were higher than the predicted χ^2 values (Table 1), indicating a greater deviation of the response data from the regression lines, i.e. the experimental data for these two strains were a poor fit for the model used by ViStat [16].

Discussion

Making direct comparisons between the results obtained in this paper and those reported in previous studies is somewhat problematic, because the bioassay methodologies used vary considerably. Unlike many other studies of gypsy moth virus strains, in which the lethal concentrations (LC) for different isolates of LdMNPV were determined, this current and one of the previous studies [16] determined the more accurate lethal doses (LD) of three isolates of LdMNPV. Interpreting the results of some of the earlier studies using LC is made even more difficult because, in some cases, cytoplasmic viruses were also tested at the same time [30, 31]. Earlier studies also reported variations in the biological activities of different geographic isolates of gypsy moth virus [30-35], and variation in the responses of gypsy moth larvae from different geographic locations to the same virus strain. The age or phase of the gypsy moth outbreak, the isolation, preparation and storage of the strains are also thought to influence the biological activity (both pathogenicity and virulence) of the virus [33].

A previous study [16], using the contaminated diet plug method, compared the dose and time-responses of second-instar larvae of the EGM and AGM-R to three gypsy moth virus isolates. Both experiments used almost identical methodologies, the only difference being that the field-collected larvae used for the bioassay in China were starved for 24 h prior to inoculation, whereas the larvae used in the Canadian study were from laboratory colonies and were not starved. Because we tested the same three virus strains against second-instar AGM-C larvae using an almost identical methodology, most of comparisons will be between the results we report in this paper and the

previous study. Hence, we presented our data using the same format (Table 1) as in Ebling et al. [16] to facilitate comparison of the bioassay results.

Comparison of the pathogenicity of three LdMNPV isolates against AGM-C and AGM-R

When second instar AGM-C larvae were challenged with the three virus isolates, we found that the pathogenicity of LdMNPV-D and LdMNPV-H against AGM-C was not significantly different. However, both LdMNPV-D and LdMNPV-H were significantly more pathogenic against AGM-C than LdMNPV-J.

In comparison, Ebling et al. [16] reported that LdMNPV-H was more pathogenic against AGM-R than LdMNPV-J, and significantly more pathogenic than LdMNPV-D. LdMNPV-H had a significantly lower LD_{50} (648 OBs $larva^{-1}$) than LdMNPV-J (1260 OBs $larva^{-1}$) and LdMNPV-D (1904 OBs $larva^{-1}$). The LD_{95} of LdMNPV-H (8540 OBs $larva^{-1}$) was significantly lower than that of LdMNPV-D (208 600 OBs $larva^{-1}$), but not for LdMNPV-J (20 841 OBs $larva^{-1}$). Comparing the pathogenicity of the three virus isolates against AGM-C (this study) and AGM-R [16], LdMNPV-D and LdMNPV-H were significantly more pathogenic against the AGM-C than to the AGM-R, while LdMNPV-J was somewhat more pathogenic against the AGM-C than to the AGM-R, but not significantly.

Comparison of the virulence of three LdMNPV isolates against AGM-C and AGM-R

Interesting patterns emerged when we compared the time-responses (virulence) of the same three virus isolates against AGM-C and AGM-R. There were no significant differences among the median time-responses (ST_{50}) of the three virus isolates (LdMNPV-D, LdMNPV-H and LdMNPV-J) when second-instar AGM-C larvae were challenged (Table 1). Similarly, there were no significant differences among the three virus isolates in the time-responses when second-instar AGM-R larvae were challenged [16]. The ST_{50} of AGM-R to the three virus isolates varied from 10.4 to 11.5 d [16]. These ST_{50} values are about 1.5 to 2 d longer than what we obtained for AGM-C, which varied from 8.3 to 8.9 d (Table 1).

Possible sources of variability in virus activity

LdMNPV-D, the active ingredient in the registered virus products in North America (Gypchek and Disparvirus®), was significantly more pathogenic against the EGM than the two strains of AGM bioassayed. It has been postulated that this virus strain was accidentally introduced into North America, either with its host or with one of the introduced parasitoids of gypsy moth [36]. Thus, it is highly likely that LdMNPV-D virus strain has always been associated with the EGM.

LdMNPV-H and LdMNPV-D were equally pathogenic against AGM-C, and significantly more than LdMNPV-J. The fact that LdMNPV-D and LdMNPV-H were equally effective was unexpected, because one would have expected that LdMNPV-H, isolated in Heilongjiang Province in China, to be more pathogenic to *L.d. asiatica*. Likewise, it was somewhat surprising that LdMNPV-J was the least pathogenic strain against AGM-C (Table 1), and only moderately pathogenic against AGM-R [16], unless it was originally isolated from a species of Lymantriid other than the Japanese gypsy moth (*L.d. japonica*).

Previous studies (using LC_{50}) have revealed differences in pathogenicity among geographic isolates of LdMNPV. One study [37] found that the LdMNPV isolate from France was less active compared with a North American and a Korean isolate. In a different study [38], six geographic isolates of LdMNPV from China were tested against EGM established in eastern North America. None of these virus strains were found to be as effective as the strain used in

the currently registered products (Gypchek and Disparvirus®) for gypsy moth control in North America. In a third study [34], 19 different strains of LdMNPV were bioassayed against a laboratory colony of the introduced EGM, including one strain from Japan. The median lethal concentration (LC_{50}) values of the geographical isolates against the colonized strain of *L. dispar* in the US varied from 1.7×10^3 to more than 5×10^6 OBs ml^{-1}. It was also found the North American virus isolate was generally the most active against gypsy moths, and the virus from Japan was the least active of the isolates tested. Unfortunately, the origin of the Japanese virus strain tested was not indicated [34], and when they did their work the gypsy moth complex was not stated.

As previously mentioned, it is also possible that the nucleopolyhedrosis virus from Japan was not isolated from *L.d. japonica*, but from one of the other species of *Lymantria* that are known from Japan. Recent taxonomic work [2] proposed that what was once considered a single species (*L. dispar*) actually consists of several species and subspecies, five of which occur in the Kurile Islands, Japan, and Ryukyu Islands. Interestingly, the AGM, *L.d. asiatica*, was not listed as one of the gypsy moths occurring in Japan [2]. Despite repeated attempts, we could not precisely pinpoint either the location or the scientific name of the host from which the LdMNPV-J strain we used was obtained. However, we think that it is highly probable that the virus strain we used was obtained from *L.d. japonica* (Motschulsky), the most widely distributed subspecies of gypsy moth in Japan.

These variances may also be due to differences in larval strains of AGM used in the two studies. The AGM-R larvae tested during the previous study [16] were reared from a laboratory colony of a Russian strain obtained from the Otis Methods Development Center, USDA, APHIS, PPQ, Massachusetts, that had been in rearing for over 51 generations (John Tanner, USDA, personal communication), while the AGM-C larvae tested in our experiment were collected from the field in He Ling County near Huhhot in north-western China. Thus, the differences in the experimental results may also be due either to changes in resistance in the AGM-R somehow induced by prolonged breeding under laboratory conditions, compared with the field-collected AGM-C, or simply to comparing different strains of the same insect. It would be interesting to see if the biological activity of these same three geographic strains of virus would be different if tested (using the same methodology) against a field-collected Russian and Japanese strains of Asian gypsy moths.

It has been shown by this study and others that both the geographic isolate and the gypsy moth larval strain tested influence the effectiveness of the virus as a biopesticide. Therefore, it is very important to select the most virulent isolate for a given host population, first by laboratory bioassay, then by confirmation in small-scale field tests, before seeking registration or proceeding with large-scale field use as a biopesticide. However, this is not always practical in operational gypsy moth, or other defoliator, control programs, if the virus product is not already registered.

The AGM-R tested in the previous study [16] and the AGM-C in our current study occur in the two geographic areas from which the introduction of *L.d. asiatica* to North America is most likely to originate due to the ever-increasing trade with these two countries. There are two other potential sources for introduction from Asia, Japan and Korea. Given the differences in the dose and time responses observed in the AGM-R challenged in the previous study [16] and in this study (AGM-C), it would be highly desirable to test these same three gypsy moth virus strains against the Japanese gypsy

moths identified as native to Japan [2], or at the very least against *L.d. japonica* (the most common subspecies). This subspecies, with its wide distribution in Japan, may be a third possible source of introduction of AGM into North America.

Recent DNA work on Lymantriid moths [39] generally agrees with the earlier work by Pogue and Schaeffer [2], but also states that *L.d. asiatica* and *L.d. japonica* cannot always be easily separated. Increasing trade among Asian countries, with its potential for introductions could have further complicated the distinction between *L.d. asiatica* and *L.d. japonica* (LM Humble 2010, personal communication). It is recommended that the same three virus strains be bioassayed against recently collected strains of *L.d. asiatica* from the Russian Far East, China, and Korea, as well as *L.d. japonica* from Japan. DNA barcoding of the field-collected AGM strains being bioassayed would also be desirable, especially in light of possible past misidentifications of some *Lymantria* spp. [40]. The other, less widely distributed gypsy moths of the subgenus *Porthetria* in Asia, *Lymantria albescens, L. postalba*, *L. umbrosa,* and *L. xylina*, could be studied at a later date. Bioassaying the species or subspecies of gypsy moth occurring in Japan could be done cooperatively with researchers in Japan to avoid quarantine concerns and accelerate this research.

Acknowledgements

Thanks are extended to Mr. Te Muqin, Mr. Gang Song and Mr. Shengguo Zhao, Inner Mongolia Forestry Protection Station, for providing the Asian gypsy moth egg masses; and to Xiaoqin Wang, Lichun Zhu and Yuye Gong for their assistance bioassaying the Chinese strain of Asian gypsy moth. Funding for the bioassay and publication costs was provided by the Canadian Forest Service, Natural Resources Canada. Dr. Chris Lucarotti and Mr. Peter Ebling, both from the Canadian Forest Service, provided constructive comments on an earlier version of this manuscript. We would also like to thank the anonymous reviewers for their valued comments on the earlier draft of this manuscript.

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DEVELOPMENT OF FOREST PROTECTION BIOLOGICAL OPERATIONS AGAINST INVASIVE ORGANISMS

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摘要：新的外来入侵生物多滋扰俄罗斯森林，使森林面临着极大的威胁，造成大面积的森林被毁灭。例如复叶槭（*Acer negundo*）、美国红梣（*Fraxinus pennsylvanica*） 刺槐（*Robinia pseudoacacia*） 等外来植物正入侵俄罗斯森林。一些林缘、林中空地、皆伐和火烧林地常被琐氏独活（*Heracleum sosnowskyi*）、蓼属植物（*Polygonum weyrichii*）、多叶羽扇豆 *Lupinus polyphyllus*）等许多外来入侵种覆盖。近期入侵我国森林的白蜡窄吉丁（*Agrilus planipennis*）、冷杉四眼小蠹（*Polygraphus proximus*）和黄杨木蛾（ *Cydalima perspectalis*）已被列为极其危险的有害生物。近年在俄罗斯南部森林发现的危害松树种籽的欧美喙缘蝽（*Leptoglossus occidentalis*）对欧洲和西伯利亚地区的森林种子生产造成巨大威胁。此外，还有一些食虫类昆虫对早期引进俄罗斯的刺槐造成危害，这些物种已经扩散蔓延到了俄罗斯南部林区的10%左右面积。如果像刺槐叶瘿蚊（*Obolodiplosis robinea*）、刺槐潜叶蛾（MINERS *Parectopa robiniella* ）和刺槐潜细蛾（*Phyllonoricter robiniella*）大规模爆发将会对大片的俄罗斯森林造成破坏。

每一个生物入侵种都需要发展一种针对性的森林保护策略来抵抗新的外来生物。令人遗憾的是，我们对许多外来入侵种尤其是它们对森林的风险还认识不足。

关键词：外来入侵生物，森林保护

РАЗРАБОТКА МЕР БИОЛОГИЧЕСКОЙ ЗАЩИТЫ ЛЕСА ОТ ИНВАЗИВНЫХ ОРГАНИЗМОВ

В леса России все чаще проникают новые инвазивные организмы, некоторые из которых становятся опасными и наносят существенный ущерб. В леса страны проникли такие сорные растения, как клен ясенелистный *Acer negundo*, ясень пенсильванский *Fraxinus pennsylvanica*, робиния псевдоакация *Robinia pseudoacacia* и др. По опушкам, полянам, вырубкам и гарям российских лесов все чаще успешно разрастаются такие чуждые для наших лесов пришельцы, как борщевик Сосновского *Heracleum sosnowskyi*, горец Вейриха *Polygonum weyrichii*, люпин *Lupinus polyphyllus* и многие др. К числу наиболее опасных вредителей следует отнести недавно появившихся у нас ясеневую узкотелую изумрудную златку *Agrilus planipennis*, уссурийского короеда *Polygraphus proximus* и самшитовую огневку *Cydalima perspectalis*. Не так давно в лесах юга России выявлен также сосновый семенной клоп *Leptoglossus occidentalis*, который может представлять большую опасность для лесосеменного дела в европейской части страны и на юге Западной Сибири.

На территории России появилось также несколько фитофагов, повреждающих ранее завезенную белую акацию *Robinea pseudoacacia.* В

некоторых районах юга России эта порода в настоящее время занимает порядка 10% лесопокрытой площади и формирование очагов массового размножения белоакациевой листовой галлицы *Obolodiplosis robinea* и минеров *Parectopa robiniella* и *Phyllonoricter robiniella* может нанести существенный ущерб древостоям.

Каждый случай инвазии требует разработки собственной стратегии защиты леса от нового вселенца. К сожалению, опасность проникновения мнгоих видов, особенно сорных растений, для леса в настоящее время еще не достаточно оценена.

Ключевые слова: инвазивные организмы, защита леса.

ABSTRACT: New invasive organisms more often infest Russian forests and some of them become hazardous and bring severe damage. Such weed plants as *Acer negundo*, *Fraxinus pennsylvanica*, *Robinia pseudoacacia* and others penetrated our forests. Russian forest woodsides, openings, cutover and burned out areas more often successfully overgrow with such foreign invades as *Heracleum sosnowskyi*, *Polygonum weyrichii*, *Lupinus polyphyllus* and many others. *Agrilus planipennis*, *Polygraphus proximus* and *Cydalima perspectalis* that infested our forests recently can be rated as the most hazardous pests. Lately pine seed bug *Leptoglossus occidentalis* was found in south Russia forests it can be a big challenge for forest seed production in European Russia and West Siberia south.

Also some entomophages that affect imported earlier *Robinea pseudoacacia* infested Russian territory. At the moment in some south Russia regions this species cover around 10% forest covered area so development of *Obolodiplosis robinea,* miners *Parectopa robiniella* and *Phyllonoricter robiniella* mass outbreaks may damage the woods sufficiently.

Each invasion case needs development of forest protection individual strategy against a new invader. Unfortunately risks of many species invasion in particular weed plants for forests are underestimated yet.

Key words: invasive organisms, forest protection.

Introduction

Occurrence of new invasive plant species as well as insects and agents of tree-brush species diseases in Russian forests is a common case. Such weed plants as *Acer negundo*, *Fraxinus pennsylvanica*, *Robinia pseudoacacia* etc. infested forests in our country. Such foreign in our forests invaders as *Heracleum sosnowskyi*, *Polygonum weyrichii*, *Lupinus polyphyllus* and many others more often successfully grow in edges, clearings and burnt areas in Russian forests. So far invasion risk of foreign tree-brush and grass plants in our forests has not found proper awareness among our foresters. However occurrence of new harmful insects and diseases attracts greater attention since it needs fast and efficient response to the damage. But often efficient operations are not feasible since biological specifics of these hazardous organisms in its new habitats are unknown and protection procedures are not developed by its occurrence.

This paper goal is to highlight state of art in biological forest protection against key invasive insects and forest diseases agents in Russia.

The study focuses on a number of invasive organisms and related entomophages that infested Russian forests. .

Problem solution. Globalization of world economy and intensive integration of Russia in it resulted in sufficient growth of new foreign to native forest communities organisms that have already infested or can invade them. Thus

around 15 invasive insect species (table 1) and 10 tree species disease agents (table 2) have already infested the Russian territory since early XXI century.

Many of them are not a great hazard for forests but some have shaped a principally new reality when its infestation results in not only native community transformation but a hazard for some forest community types and some tree plant existence.

Such recent organisms as *Agrilus planipennis*, (Mozolevskaya, Ismailov, 2007), (Mozolevskaya et al, 2008), *Polygraphus proximus* (Gninenko et al, 2010a, 2010b), *and Cydalima perspectalis* (Karpun et al, 2014, Shurov, 2014) can be referred to the most hazardous pests. Lately the pine seed bug *Leptoglossus occidentalis* was identified in South Russian forests it may be a great hazard for seed production in European Russia and south West Siberia (Gapon, 2012, Gninenko et al, 2014).

Pic. 1. Severe damage of boxwood bark by *Cydalima perspectalis* caterpillars.

Practically right after its infestation *Cydalima perspectalis* of new habitats in boxwood forests in Russian Black Sea coast it drastically damaged natural boxwood forests as well as greenery plantations (pic. 1).

Emerald ash borer damage in community ash plantations is great. Now it started infestation of natural ash woods where it shapes mass outbreaks.

Polygraphus proximus and transmitted pathogene fungus *Grosmannia aoshimae* that infested Siberian fir woods resulted in fir wood mortality in a number of Siberian regions in area over 100 thousand ha (Kravets et al, 2015).

Development of protection operations against new invaders is always late. It is an objective difficulty however time lag can be cut.

Table 1. **Most hazardous insect invaders in Russian forest communities**

Species name	Infesstation time in Russia	Origin
Hyphantria cunea	60-es, XX century	North America
Corythucha ciliata	1998	North America
Cameraria ohridella	2003	West Europe
Obolodiplosis robiniea	2005	North America
Parectopa robiniea	2007	North America
Agrilus planipennis	2003	Far East
Phylonorycter issikii	80-es, XX century	Far East
Leptoglossus occidentalis	2012	North America
Phylonorycter robiniella	early XX1 century	North America
Aproceros leucopada	early XX1 century	East Asia
Metcalfa pruinasa	early XX1 century	North America
Cydalima perspectalis	2012	East Asia

Table 2. **Some agents of forest tree brush species diseases that infested Russian forest communities**

Species name	Infesstation time in Russia	Origin
Agents of *Ophiostoma ulmi* *Ophiostoma novo-ulmi*,	30-es, XX century 50es, XX century	North America
Ageent of *Mycrosphera alphitoides*	Early XX century	North America
Agent of *Erysiphe flexuosa*	2003 .	North America
Agent of *Cryphonectria parasitica*	1908 .	East Asia
Grosmannia aoshimae	early XX1 century	East Asia
Phytophthora alni	2010	West Europe
Lophodermium nitens	2007	North America

For the 1st time often new invaders occur in big cities and transport centers. Thus *Polygraphus proximus* for the 1st time was identified in Saint-Petersburg sea terminal area and then in Moscow vicinity (Mandelstam, Popovichev, 2000, Chlakhsaeva, 2008), *Cydalima perspectalis* infested Russia in Sochi territory and started its spread from there (Gninenko et al, 2014) and *Corythucha ciliata* for the 1st time was found in Krasnodar (Voigt, 2001).

Practically in such conditions it is impossible to carry out fast and total suppression of the identified pest with available pesticides since there are strict restriction on toxic chemical application.

Thus development of its biological suppression operations is a key area to protect against invasive organisms. .

More comprehensive knowledge of its biology specifics in new habitats enables development of biological protection operations against a new hazardous organism. Thus it is desirable to study a new invader biology and search its entomophages and pathogenic microorganisms in collaboration with foreign colleagues as it occurs in neighbor states of Russia.

Studies of new invader species biology and parallel search of efficient and safe protection against them is under way at VNIILM now.

Application of the pupae parasitoid *Chouioia cunea* started as a protection operation against *Cydalima perspectalis.* This eulophid parasite laboratory rearing technology on various host species has been developed and releases in *Cydalima perspectalis* mass outbreaks in Sochi area are under way.

Studies of emerald ash borer and its parazitoids are ongoing for several years. Now new local entomophages that began efficient regulation of its population have been identified. It enables preconditions to hope that risks for ash can be sufficiently reduced not only in Russia but in European states as well.

Horse chestnut integrated protection system against Ochrid miner has been under development over several years. Now there are proposals that provide use of Horse chestnut species resistant to the pest in greenery plantations, pheromone trap application, removal of falling leaves during leaf fall, pesticide applications where allowable and other operations.

Studies to develop fir protection operations against *Polygraphus proximus* and related pathogenic fungus are under way.

We believe we should start development of seed facility protection operations against pine seed bug since there are proofs that it promotes pine infestations with hazardous myscromycetes.

Several phytophages that affect *Robinea pseudoacacia.*occurred in Russian territ*ory.* Now in some south Russian areas this species accounts for around 10% of forest covered area and development of *Obolodiplosis robinea*

and miners *Parectopa robiniella* and *Phyllonoricter robiniella* can damage available woods sufficiently.

Conclusions. The studies enabled identification of a great number of invasive organisms that not only infested Russian forest communities but became hazardous pests.

Maximum fast study of these new forest resident biology, search of its efficient entomophages, its production and application technology is needed for development of biological protection operations against new invaders.

International cooperation aimed at timely invader identification and development of protection operations against contributes greatly to successful protection against invasive organisms.

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VIRUS APPLICATION EXPERIENCE TO PROTECT BOXWOOD AGAINST *CYDALIMA PRESPECTALIS* CATERPILLARS

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利用病毒保护黄杨木以抵御盒树蛾

摘要：本文鉴定了索契地区黄杨木蛾（*Cydalima prespectalis*）自然种群中的病原病毒菌株。并利用欧洲松叶蜂（*Neodiprion sertifer*）核型多角体肠道病毒（neovir生物制剂）和舞蠹蛾核型多角体病毒（pinkvir）对黄杨木蛾进行了生物活性测定。研究结果表明，外源病毒也对黄杨木蛾具有高的致病性，也可应用于黄杨木的保护。

关键词：昆虫病原病毒，黄杨木蛾

ОПЫТ ИСПОЛЬЗОВАНИЯ ВИРУСОВ ДЛЯ ЗАЩИТЫ САМШИТА ОТ ГУСЕНИЦ САМШИТОВОЙ ОГНЕВКИ

Приведены данные по смертности гусениц инвазивного вредителя самшита – самшитвой оневки от чуждых вирусов. Испытаны вирусы ядерного полиэдроза кишечного типа рыжего соснового пилильщика (неовир) и вирус ядерного полиэдроза гусениц рода *Lymantria* (пиквир). Показано, что чуждые вирусы вызывают высокий уровень смертности гусениц самшитовой огневки и их возможно использовать для защиты самшита от этого опасного вредителя.

Ключевые слова: энтомопатогенные вирусы, самшитовая огневка.

ABSTRACT: Result data of tests of entomopathogenic viruses in *Cydalima prespectalis* natural populations in Sochi area. Red pine sawfly *Neodiprion sertifer* nuclear polyhedrosis intestine virus (neovir biological agent) and general *Lymantria* nuclear polyhedrosis virus (pinkvir) were tested. It was found that foreign viruses can ensure high *Cydalima prespectalis* caterpillar mortality rates which enables its application in boxwood protection.

Key words: entomopathogenic viruses, *Cydalima prespectalis*

Introduction.

Cydalima perspectalis Walker, 1859 (Lepidoptera: Pyraloidea: Crambidae) – is an Asian species for the 1st time in Europe found in 2006 in Germany and in 2007 was put in the EPPO alert list (Alert list EPPO). However its expansion across European countries was so rapid that its quarantine control viability was called in question so in 2011 the pest was drawn out of the alert list.

Cydalima perspectalis entered the Russian territory in 2012 with greenery plants imported from Italy (Gninenko et al. 2014; Karpun et el., 2014; Schurov, 2014). In the Krasnodarsky territory in natural boxwood forests and greenery

plantations it expanded rapidly with serious boxwood impacts everywhere. In fact by 2015 the pest was identified across the whole boxwood (*Buxus* sp.) areal including the Stavropolje and Crimea however so far there is no efficient protection resources against *Cydalima perspectalis.*

Material and procedures.

Initially we tried to find in *Cydalima perspectalis* populations its own virus. We undertook search of its dead bodies that occurred in mass as a result of lack of fodder in boxwood forests in Sochi area (Krasnodarsky territory). Its caterpillars completely defoliated all boxwood crowns here in 2014. Part of them died of lack of fodder since it could not finish feeding on other forest plants. Similar situation was in some areas in 2015. Malnutrition attempts to start feeding on unsuitable plants could promote evolution of a latent virus infection and trigger caterpillar virosis-related mortality.

Collected dead bodies were brought to the laboratory and analyzed for available entomopathogenic microorganisms in its tissues.

Virus particles (polyhedral) that might trigger its mortality were identified in soma dead bodies. Such dead bodies were homogenized in a mortar and the homogenate was initially treated. Due to risk of virus loss since its number in suspension was relatively low complete treatment was not conducted. This suspension was applied in the follow-up sprayings. Experiment was conducted on boxwood branch bunches sprayed with the prepared suspension. 29 caterpillars were planted on each bunch. The control bunch was sprayed with water.

It is known that application of *Anagrapha falcifera* foreign virus enabled rather high *Cydalima perspectalis* caterpillar mortality rate (Rise et all, 2013 a,b). Thus we picked 2 available viruses: *Neodiprion sertifer* intestine nuclear polyhedrosis virus (Neovir biological preparation) and *Lymantria* common nuclear polygedrosis virus (Pinkvir). 1x 10^9 titre virus suspensions were prepared and boxwood branch bunches sprayed. In 1 hour after spraying 2-3 age caterpillars were planted on the brunches. After total finish of the treated fodder boxwood untreated branches were given for caterpillar feeding. The bunches were kept at temperature +20 – +22ºC, humidity around 70% and natural light period duration.

Caterpillar condition was based on daily visual survey findings. Feeding intensity by excrement volumes and caterpillar mortality were registered.

Registration was stopped on the 15th day after spraying.

In lab conditions we managed to test impacts of some environmental preparations on *Cydalima perspectalis.* The commercial preparations based on Dimilin as wellm as Gypsy moth (Neovir) and red pine sawfly (Pinkrivin) nuclear polyhedrosis viruses were applied in our experiments. Single application of 0,1 and 0,5% dimilim solutions against 2-3 age *Cydalima perspectalis* caterpillars resulted in practically total appetite loss during initial 48 – 72 hours after treatment and 100% mortality in the following 3 weeks without any visible impacts on protected plants. In result of single virus preparation treatments small part of 2-3 age caterpillars (around 4% after “Neovir” and around 6% after”Pinkvir”), managed to reach imago stage however plant damage rate by the pest in these cases was 5 – 7 lower compared to the control (Abasov et al, 2016).

Findings and discussion.

The microbiological analysis of *Cydalima perspectalis* caterpillar dead bodies found in Sochi area woods showed that a higher number of bacteria that triggered infection process evolution were observed more often (table. 1).

Table 1. ***Cydalima perspectalis*** **dead caterpillar analysis findings found in its mass outbreaks**

Total number of analyzed found caterpillar dead bodies, pcs.	Caterpillar share (%%), killed by		
	bacteria	viruses	Other agents
15	66.7	13.3	20.0*

*Note: This column takes into consideration caterpillars featuring damage by other caterpillars namely most likely died of cannibalism.

Rather great number of caterpillars had impacts featuring cannibalism cases. In lack of fodder conditions *Cydalima perspectalis* cannibalism is a common case.

Polyhedra were found only in 13.3% caterpillars. However it were not numerous and we believe that it was not only a key but an associated caterpillar mortality factor.

Thus on mass mortality background due to lack of fodder its rather relative small part died with available virus inclusions in cells.

4 and 5 age caterpillars were used in experiment. Suspension treatment resulted in mortality of only 16.7% caterpillars involved in the experiment. It proves that *Cydalima perspectalis* baculovirus is likely unable to reproduce rapidly so it can not trigger virus epizooty evolution in natural conditions. Dead caterpillar bodies analysis that found lack of virus polyhedral in dead bodies proves this preliminary conclusion. Thus caterpillars were killed by other factors rather than virus infection. This experiment showed that there is no efficient virus able to trigger active epizooties in *Cydalima perspectalis* population in its mass outbreaks in Sochi area. It makes virus biological agent development based on *Cydalima perspectalis* own virus more complicated.

In literature sources there is information on effect of *Anagrapha falcifera* virus applications (Rise et all, 2013 a,b) against *Cydalima perspectalis* caterpillars. For application opportunity testing we used *Neodiprion sertifer* intestine nuclear polyhedrosis virus (Neovir biological preparation) and *Lymantria* common nuclear polygedrosis virus (Pinkvir) (table 2).

Table 2. **Laboratory testing results of some agents to protect boxwood against *Cydalima perspectalis* caterpillars**

Preparation name	Active substance	Application results	
		Mortality termination (days after spraying)	Dead caterpillar share , %
Neovir	Red pine sawfly nuclear polyhedrosis virus	15	96.0
Pinkvir	*Lymantria* nuclear polyhedrosis virus	15	94.0

Thus testing of viruses foreign for *Cydalima perspectalis* found that despite long caterpillar mortality period final early age caterpillar mortality was high. It enables looks for boxwood protection procedure development opportunity based on application of viruses foreign for *Cydalima perspectalis.*

Mechanism of foreign virus impacts on *Cydalima perspectalis* caterpillars is still understudied and should be a key area in boxwood protection technology development.

Conclusion.

The studies in *Cydalima perspectalis* mass outbreaks showed lack an active virus able to trigger virosis epizooty in its populations.

2 foreign viruses testing showed an opportunity of high *Cydalima perspectalis* caterpillar mortality that makes this phytophage caterpillar control virus application technology development feasible.

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GENOMIC SEQUENCE ANALYSIS OF *HELICOVERPA ARMIGERA* NUCLEOPOLYHEDROVIRUS ISOLATED FROM AUSTRALIA

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摘要：本文测定和分析了一株来自澳大利亚的棉铃虫核型多角体病毒（HearNPV-Au）全基因序列。HearNPV-Au基因大小为130,992bp，G+C含量为39mol%，含有134预测开放阅读框架(ORFs)和超过150个核苷酸组成。HearNPV-Au毒株的94 ORFs与苜蓿银纹夜蛾核型多角体病毒（AcMNPV）、棉铃虫核型多角体病毒单粒包埋型（HearSNPV-G4）和棉铃虫核型多角体病毒多粒包埋型（SeMNPV）比较，更加接近HearSNPV-G4毒株。证明HearNPV-Au和HearSNPV-G4基因组之间的核苷酸序列有99%同源。发现它们的主要区别在同源区域(*hrs*)和杆状病毒重复的ORFs区(*bro*)基因。5个*hrs*和两个bro基因证明存在于HearNPV-Au基因组中。其中，除了在HearSNPV-G4中同源的ORF59外(*bro*)HearNPV-Au中的全部134个ORFs确定在HearSNPV-G4中也被发现。基因序列数据充分证明HearNPV-Au和HearSNPV-G4属于同一种病毒。

关键词：棉铃虫核型多角体病毒，基因，全序列分析

АНАЛИЗ ГЕНОМА ВИРУСА ЯДЕРНОГО ПОЛИЭДРОЗА *HELICOVERPA ARMIGERA* АВСТРАЛИИ

Показаны результаты секвенирования генома вируса ядерного полиэдроза хлопковой совки *Helicoverpa armigera* из Австралии (HearNPV-Au). Высокая генетическая вариабильность вируса ядерного полиэдроза хлопковой совки известна. Результаты показали, что изолят HearNPV-Au на 99% идентичен типовому изоляту HearNPV-G4, что свидетельствует об их принадлежности к одному виду.

Ключевые слова: вирус ядерного полиэдроа хлопковой совки, гены, анализ генома вируса.

ABSTRACT: The complete genomic sequence of *Helicoverpa armigera* nucleopolyhedrovirus from Australia, HearNPV-Au, was determined and analyzed. The Hear-NPV-Au genome was 130,992 bp in size with a G+C content of 39 mol% and contained 134 predicted open reading frames (ORFs) consisting of more than 150 nucleotides. HearNPV-Au shared 94 ORFs with AcMNPV, HearSNPV-G4 and SeMNPV, and was most closely related to HearSNPV-G4. The nucleotide sequence identity between HearNPV-Au and HearSNPV-G4 genome was 99 %. The major differences were found in

homologous regions (hrs) and baculovirus repeat ORFs (bro) genes. Five hrs and two bro genes were identified in the Hear-NPV-Au genome. All of the 134 ORFs identified in HearNPV-Au were also found in HearSNPV-G4, except the homologue of ORF59 (*bro*) in HearSNPV-G4. The sequence data strongly suggested that HearNPV-Au and HearSNPV-G4 belong to the same virus species.

Key words: *Helicoverpa armigera* nucleopolyhedrovirus, gene, complete genomic sequence analyzed

Introduction

Being pathogenic to many insects, baculoviruses are frequently used as bio-insecticides to control the size of pest populations in nature. It is well established that baculovirus populations exhibit large amounts of genotypic variation, which may have multiple origins, such as geographical or temporal differences, a different host, or even an individual host [1–8]. The cotton bollworm *Helicoverpa armigera*,is a serious global pest that is responsible for economic losses to over 60 cultivated crops and is resistant to chemical insecticides. In China, HearNPV (family *Baculoviridae*, genus *Alphabaculovirus*) has been commercialized and extensively used on cotton fields since 1994 [9, 10]. High levels of genetic variation have also been found within HearNPV populations [6–8]. Now, genomes of five *Helicoverpa* spp. NPVs, including HearSNPV-C1 (China) [11], HearSNPV-G4 (China) [12], HearNPV-NNg1 (Kenya) [13], HzSNPV (USA) [14], and HearMNPV (China) [15], have been sequenced. The gene content and arrangement of HearMNPV were distinct from the other four NPVs, and those four NPV genomes shared very high nucleotide sequence identity except for the homologous regions (hr) and the baculovirus repeat ORFs (bro). In this study, we sequenced and analyzed the complete genome of another HearNPV, HearNPV-Au, which isolated from Australia.

Materials and methods

The HearNPV-Au used in this study was supplied by Tri-Delta Chemicals Pty Ltd. (Australia) and Henan Jiyuan Baiyun Industry Co., Ltd. (China). Polyhedra of HearNPV-Au were propagated in *H. armigera* larvae and purified by washing with 1% SDS and distilled water multiple times with centrifugation. The purified polyhedra were solubilized in 0.7 ml alkaline solution (0.1 M Na2CO3, 0.1 M NaCl, 0.005 M EDTA, pH 8.0) at 37℃ for 1 h. The pH was adjusted to 7.0 with 0.1 M HCl, 5μl of 20 mg/ml proteinase K was added and the sample was incubated at 55℃ for 3 h. The genomic DNA was extracted with phenol and chloroform, precipitated with 100 % ethanol, and washed with 70 % ethanol.

A random genomic library of HearNPV-Au was constructed according to the ‘‘partial filling-in’’ method as described previously with minor modifications [16]. Viral DNA fragments ranging from 1.5 to 5.0 kbps were cloned into the *Sal*I site of the pUC19 vector. A total of 464 recombinant plasmids were prepared for sequencing using a BigDye Terminator v3.1 Cycle Sequencing Kit (ABI) on a Genetic Analyzer 3130XL (ABI). The combined sequence generated from these clones represented sixfold genomic coverage. Additional sequences for conformation of ambiguous regions and for filling in of gaps in the assembled sequence were obtained from sequencing of PCR products. All of the sequences were assembled into contigs using SeqMan from the DNASTAR 7.0 software package.

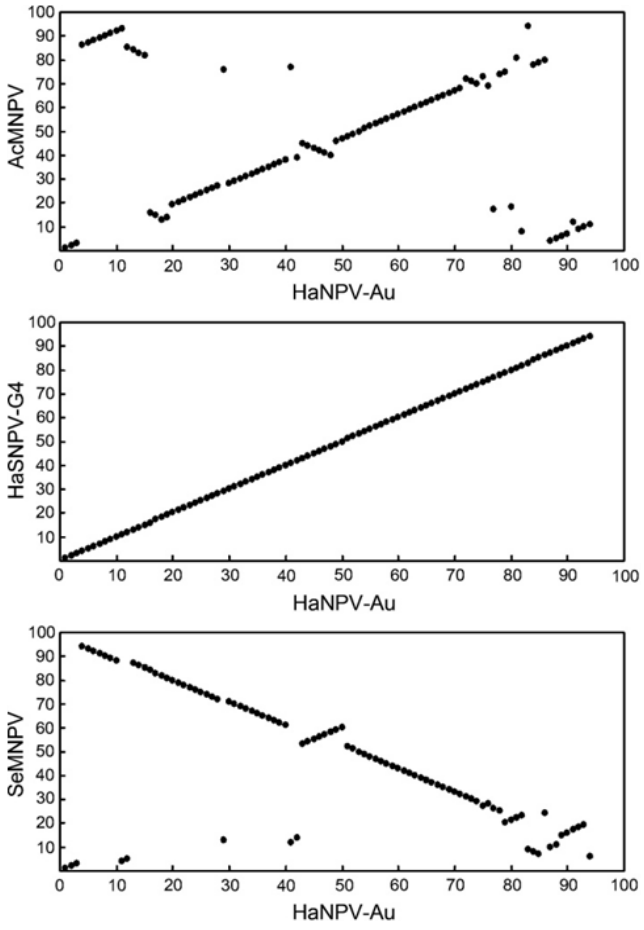
ORFs were defined using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). DNA and protein comparisons were performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or Vector NTI Advance Suite v8.0. Multiple sequences were aligned in Clustal X and displayed in GeneDot. Promoter motifs present upstream of the putative ORFs were screened as described previously [17].

Results and discussion

During the assembly of the genome sequence, we found some nucleotide variability. Based on a longest assembled sequence (13.5 kb), the rate of nucleotide variability was 0.19 %. We just picked the predominant nucleotide when nucleotide variability occurred. This confirmed that Hear-NPV populations exhibit genotypic variation.

The HearNPV-Au genome was 130,992 bp in size(GenBank accession no. JN584482), similar to those of HzSNPV (130,869 bp, GenBank accession no. AF334030) and HearSNPV-G4 (131,405 bp, GenBank accession no. NC002654), with a G + C content of 39 mol%. There were 134 predicted ORFs consist of more than 150 nucleotides. The HearNPV-Au genome shared 94 ORFs with the AcMNPV, HaSNPV-G4 and SeMNPV genomes. Homologues of these 94 ORFs were chosen for the GeneParityPlot analysis. The comparison showed that HearNPV-Au and HearSNPV-G4 were completely co-lin-ear and identical in their gene arrangement (Pic. 1). The comparisons between HearNPV-Au and AcMNPV, SeM-NPV were in agreement with results reported previously [12]. A comparison of the locations and predicted amino acid sequences of the 94 ORFs between HearNPV-Au and the other three baculovirus genomes (Table 1) also indi-cated that HearNPV-Au was most closely related to HearSNPV-G4.

The nucleotide sequence identity between HearNPV-Au and HearSNPV-G4 genomes was 99 %, and the major differences were found in the hrs and bro genes.



Pic. 1. GeneParityPlot comparison of HearNPV-Au with AcMNPV, HearSNPV-G4 and SeMNPV. Homologous ORFs are plotted based on their relative location in the genome. The horizontal and vertical axes indicate the relative position of each ORF

Characterized by the presence of multiple imperfect palindrome sequences, hrs may function in gene replica-tion, transcription, recombination and rearrangement events [11, 18–20]. Both the number and location of hrs from the genomes of HearNPV-Au and HearSNPV-G4 were identical. Two types of repeats, type A and type B, were found in each of the five hrs in HearNPV-Au(supplementary material). The sequence identities of the five hrs between HearNPV-Au and HearNPV-G4 were 95.8%,99.8%,98.9%,87.7%,99.9% respectively,which showed that hr4 was more variable.Basides,hr1 contained a 58-bp insertion and hr4 contained a 289-bp insertion compared with isolate G4, and neither insertion contained a type A or type B repeat. This suggested that hrs were less conserved than ORF regions in the Helicoverpa spp. NPVs.

Table 1. **Putative ORFs identitied in HearNPV-Au**

ORF	Name	Position	Length (aa)	Promotera	Homologous ORFs AcMNPV HearSNPV-G4 SeMNPV		Amino acid sequence identity to homologues (%) AcMNPV HearSNPV-G4 SeMNPV
1	*Polyhedrin*	1 →741	246	E, L, e	8 1	1	86 100 87
2	*orf1629*	738←1979	413	N	9 2	2	27 99 33
3	*pk-1*	1994→2797	267	N	10 3	3	40 99 55
4	*Hoar*	2920←5181	753	E*	4	4	93 28
5		5377→5556	59	N	5		97
6	*Hzorf480*	5724→6575	283	E*	6		99
7		6787←6942	51	N	7		98
8	*i.e.-0*	6930→7787	285	N	141 8	138	32 99 35
9	*p49*	7804→9210	468	L	142 9	137	50 99 56
10	*odv-e18*	9221→9466	81	L	143 10	136	75 99 60
11	*odv-e27*	9481→10335	284	L	144 11	135	50 99 57
12		10381→10659	92	L	145 12	134	48 100 58
13		10686←11297	203	N	146 13	133	30 100 32
14	*i.e.-1*	11339→13306	655	E*, e	147 14	132	34 99 30
15	*odv-e56*	13359←14423	354	L	148 15	6	51 100 50
16	*me53*	14584→15663	359	E*, L	139 16-17	7	24 99 33
17		15666→15833	55	L	18		100
18		15886←16167	93	E*	19		96
19	*p74*	16188→18254	688	N	138 20	131	53 99 55
20	*p10*	18308←18571	87	L	137 21	130	18 100 51
21	*p26*	18654←19457	267	E, L	136 22	129	35 99 43
22		19570→19773	67	E*	29 23	128	32 100 48
23	*lef-6*	19849←20412	187	N	28 24	127	32 99 50
24	*Dbp*	20426←21397	323	E	25 25	126	32 100 50
25		21617→22018	133	N	26 26	125	42 100 36
	hr1	22019—24339					
26		24340←25107	255	E*	34 27	124	37 99 51
27	*ubiquitin*	24947→25198	83	L	35 28	123	75 100 78
28		25262→25768	168	E*	29		100
29	*Lese25-like*	25788→26360	190	L	30		98
	protein						
30	*39 k/pp31*	26419←27354	311	N	36 31	120	40 100 33
31	*lef-11*	27320←27703	127	N	37 32	119	39 100 51
32		27672←28388	238	N	38 33	118	52 100 63

33		28620→29699	359	E*	34		99
34	*p47*	29767←31005	412	e	40 35	115	54 99 61
35		31078→31749	223	E*	41 36		32 100
36		31835→32077	80	L	43 37	113	30 100 31
37	*lef-8*	32074←34779	901	N	50 38	112	62 99 67
38		34832→35410	192	L	51 39	111	31 99 36
39		35551→35703	50	L	40		96
40	*Chitinase*	35711←37480	589	N	126 41	19	66 98 62
41		37524←38066	180	E*	52 42	109	26 100 27
42		38184→38594	136	E, L	53 43	108	43 100 56
43		38601←39737	378	e, L	44	107	99 35
44		39745←39972	75	E*, L	45		100
45	*lef-10*	39932→40147	71	N	53a 46	106	38 100 56
46	*vp1054*	40020→41075	351	E, e	54 47	105	41 99 49
47		41195→41401	68	N	55 48	104	35 100 50
48		41402→41596	64	L	56 49	103	37 98 53
49		41875→42390	171	E, L	57 50	102	42 99 43
50		42441←42923	160	N	59 51	101	46 100 63
51		42935←43201	88	L	60 52	100	42 100 56
52	*Fp*	43413←44066	217	L	61 53	98	63 98 70
53		44238→44423	61	E	54		98
54	*lef-9*	44545→46104	519	E	62 55	97	64 99 72
55	*Cathepsin*	46188←47291	367	N	127 56	16	46 98 47
56		47332←47919	195	L	57		99
57	*gp37*	47990←48829	279	L	64 58	25	60 99 63
	hr2	48830 —49979					
58	*Bro*	49980→51629	549	N	60		96
	hr3	51630 — 52384					
59	*he65*	52385→53095	236	E*	105 61		34 100
60	*iap-2*	53172←53924	250	E, L	71 62	88	31 99 36
61		53972←54796	274	N	69 63	89	41 99 48
62		54765←55166	133	N	68 64	90	47 99 62
63	*lef-3*	55186→56325	379	N	67 65	91	25 99 32
64		56433←58790	785	L	66 66	92	29 99 61
65	*DNA pol*	58821→61883	1020	e	65 67	93	46 99 59
66		61960←62418	152	E, L	74 68		26 100
67	*HzORF384*	62484←62867	127	E, L	75 69	94	23 100 39
68		62873←63130	85	L	76 70	95	40 100 64
69	*vlf-1*	63171←64415	414	L	77 71	82	74 99 67
70		64428←64760	110	L	78 72	81	42 100 43
71	*gp41*	64829←65797	322	E*, L	80 73	80	59 100 59
72		65727←66452	241	N	81 74	79	52 100 66
73		66325←67002	225	e	82 75	78	34 99 45
74	*vp91capsid*	66932→69382	816	L	83 76	77	40 99 45
75	*cg30*	69510←70361	283	E*, L	88 77	76	27 100 33
76	*vp39capsid*	70450←71331	293	N	89 78	75	43 100 53
77	*lef-4*	71330→72715	461	N	90 79	74	44 99 50
78		72768←73532	254	N	92 80	73	53 100 59
79		73534→74022	162	N	93 81	72	55 100 63
80	*odv-e25*	74068→74760	230	e, L	94 82	71	42 100 63
81		74792←75289	165	L	83	68	98 31
82	*Helicase*	75308←79069	1253	e, L	95 84	70	42 99 47
83		79026→79547	173	N	96 85	69	48 99 63

84		79606←80571	321	N	98 86	67	45 99 53
85	*lef-5*	80467→81414	315	N	99 87	66	43 100 51
86	*p6.9*	81408←81737	109	E, L	100 88	65	43 100 67
87		81802←82911	369	L	101 89	64	40 100 51
88		82957←83325	122	E, L	102 90	63	29 100 39
89		83325←84458	377	L	103 91	62	50 99 60
90	*vp80capsid*	84553→86370	605	L	104 92	61	27 99 29
91		86367→86543	58	N	110 93	60	32 100 64
92		86558→87643	361	N	109 94	59	52 100 57
93		87688→87972	94	N	108 95	58	47 100 51
94	*odv-e66*	88039←90057	672	L	46 96	57/114	43 99 42/33
95		90078←90908	276	L	97	56	99 60
	hr4	90909 — 93506					
96		93508→94107	199	E, L	115 98	50	43 100 46
97		94111→94467	118	N	99		98
98	*Parg*	94563→96095	510	E, L	100	52	99 27
99		96174→96935	253	L	106/107 101	53	47/34 99 57
100		96950→97282	110	N	102		100
101	*iap-3*	97340←98146	268	E*, L	103	110	99 35
102		98143←98298	51	N	104		100
103	*Bro*	98409←99914	501	L	105		99
104	*Sod*	100082→100561	159	L	31 106	48	75 98 69
105		100568→101941	457	e, L	107		99
106		101994←102572	192	E, e	108		99
107		102742→103098	118	E*	109		100
108		103109→103375	88	L	117 110	47	33 100 37
109		103443→105029	528	E	119 111	36	47 99 44
110		105026→105262	78	L	112		100
111	*Fgf*	105285←106190	301	E*	32 113	38	27 100 33
112	*alk-exo*	106318←107604	428	E	133 114	41	41 99 41
113		107624←108013	129	L	19 115	42	30 100 31
	hr5	108013 — 110818					
114		109693←110619	308	E*	115a		100
115		110820→111035	71	E*	111 116		36 100
116	*lef-2*	111151←111867	238	E*	6 117	12	40 98 45
117	*p24capsid*	112229→112975	248	L	129 118	10	32 99 55
118	*gp16*	113037→113327	96	L	130 119	9	26 100 31
119	*Calyx/pep*	113379→114401	340	e, L	131 120	46	27 99 43
120		114480→114944	154	E*	63 121		26 100
121		115075→115665	196	E*, L	122		98
122	*38.7kd*	115709←116878	389	N	13 123	13	31 99 33
123	*lef-1*	116880←117617	245	N	14 124	14	38 99 47
124		117592←118020	142	E, L	125		92
125	*Egt*	118165→119712	515	E, e, L	15 126	27	44 99 52
126		119912→120490	192	N	127		100
127		120441→121241	266	E, L	17 128	29	33 99 30
128		121322←124165	947	L	129	30	99 28
129	*pkip-1*	124571→125080	169	N	24 130	32	24 97 39
130	*arif-1*	125147←125944	265	N	21 131	34	31 99 30
131		126205→127356	383	L	22 132	35	61 99 66
132		127397←129430	677	E*, L	23 133	8	26 99 39
133		129572←130117	181	E*	134		99
134		130299→130886	195	E*	135		96

Typically with multiple copies per genome, bro genes may function in nucleic acid binding, nucleosome associa-tion, and nucleocytoplasmic shuttling activity; may influ-ence baculovirus genome diversity; and are involved in recombination between baculovirus genomes [21–25]. HearNPV-Au encodes two bro genes, named bro-a (ORF58) and bro-b (ORF103) based on their gene order. HearSNPV-G4 encodes three bro genes, named bro-a (ORF59), bro-b (ORF60), bro-c (ORF105). HearNPV-Au bro-a, consisting of 1650 bp, shared 94.7% nucleotide sequence identity (96% amino acid identity) with HearSNPV-G4 bro-b and contained a 66-bp insertion compared with HearSNPV-G4 bro-b. HearNPV-Au bro-b, consisting of 1506 bp, shared 99.7% nucleotide sequence identity (99% amino acid identity) with HearSNPV-G4 bro-c. All 134 ORFs identified in HearNPV-Au were also found in HearSNPV-G4, and HearNPV-Au lacked only the homologue of another bro gene, ORF59, in HearSNPV-G4, which was the major reason for the size difference between these two genomes. This might have been caused by genetic variation in recombina-tion, suggesting that bro genes might play a role in gene exchange and evolution in different geographic locations.

Conclusions

HearNPV-Au shared 99% sequence identity with HearNPV-G4, lacking only ORF59 (bro). The sequence data strongly suggest that HearNPV-Au and HearSNPV-G4 belong to the same virus species, Helicoverpa armigera nucleopolyhedrovirus. Whether these minor differences in the genome affect pathogenicity and host range needs to be determined.

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RESISTANCE AND TOLERANCE OF FOREST STANDS TO INSECTS IN TERMS OF HOST AND FOREST SITE PREFERENCES

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基于寄主和立地参数评价森林对害虫的抵抗性和耐害性

摘要：昆虫种群动态的生物因素不仅包括天敌，也包括害虫与寄主树种的相互作用和害虫的地理区域偏好性等。

R．Painter(1953)
提出了食叶害虫与植物的相互作用的四种关系：排趋性、抗生性、耐害性和趋避性（假抗性、物候阻力）

本文旨在运用寄主树种与森林群落的抗虫性和耐虫性的知识，去论证其在害虫管理策略中的可行性。

每种食叶害虫都是以一种或者多种植物叶片为营养，其寄主范围的大小取决于地理位置、气候和大量爆发时的虫态。如具有偏好取食早期英国栎树（*Qercus robur*）的栎树卷叶蛾（*Archips crataegana* Hb.），在爆发高潮时，几乎会吃光所有树种的叶子，而在爆发结束后，又只取食偏好树种。

通过比较东乌克兰和德涅斯特河沿岸共和国的细蛾取食偏好性参数（*Phyllonory cter issikii* Kumata, 1963; *Cameraria ohridella* Deschka et Dimic, 1986; *Parectopa robiniella* Clemens, 1863），得出的结论是：这些物种的条件抗性与某些寄主植物有关。

根据种群动态的物候理论(Meshkova, 2002)，已经证明物候抗性对食叶昆虫种群动态的重要性。随着食叶害虫的宿主出现嫩叶且进入易感阶段后，害虫就会活跃并大量取食，这时害虫就会经常大爆发。当害虫处在不利的气候条件时，害虫就会处于最适合保护自己的虫态。

有扩散性的食叶害虫，其暴发常与发生地的立地条件、林龄、树冠郁闭度、树种的可食部位（松树是取食针叶、栎树是取食树叶）、林下植被、林分起源（无性繁殖或种子繁殖）、林地位置等生态条件密切相关。

森林清查数据已应用于测定食叶害虫潜在的疫区和发生区域，并用林龄作为校正预测数据的依据。

通过分析森林立地条件、林龄、林地卫生状况和爆发时期的针叶损失情况评价了松树对欧洲松叶蜂的忍受性，林地的卫生状况也许会在评价期发生变化。

关键词：森林昆虫，耐害性，食叶害虫对森林树种和林区的偏好

РЕЗИСТЕНТНОСТЬ И ТОЛЕРАНТНОСТЬ ЛЕСНЫХ НАСАЖДЕНИЙ К НАСЕКОМЫМ С ТОЧКИ ЗРЕНИЯ ПРЕДПОЧТЕНИЯ КОРМОВОГО РАСТЕНИЯ И ЛЕСНОГО УЧАСТКА

Биотические факторы воздействия на динамику популяций вредителей леса включают не только энтомофагов и энтомопатогенов, но и взаимодействие с кормовыми породами и предпочитаемость определенных лесорастительных условий и структуры насаждений.

Р. Пайнтером выделено четыре типа взаимодействия между фитофагами и кормовыми растениями: антиксеноз; антибиоз; толерантность и "ускользание" (псевдорезистентность, фенологическая резистентность).

Целью данной статьи является определение возможностей получения и использования в лесном хозяйстве знаний о резистентности к насекомым кормовых пород и участков леса и толерантности к насекомым.

Каждый вид насекомого имеет определенный набор предпочитаемых кормовых растений, который зависит от региона, погодных условий, а в очагах массового размножения насекомых – от фазы вспышки. Так боярышниковая листовертка отдает предпочтение дубу ранней формы (*Quercus robur*), по мере роста численности заселяет практически все породы, а к концу вспышки сохраняется только на предпочитаемой породе.

По данным о распространении молей-минеров (*Phyllonorycter issikii* Kumata, 1963; *Cameraria ohridella* Deschka et Dimic, 1986; *Parectopa robiniella* Clemens, 1863; *Macrosaccus robiniella* Clemens, 1859) на востоке Украины и в Приднестровье сделан вывод об условной резистентности этих видов к определенным кормовым породам.

Отмечена важная роль фенологической резистентности в динамике популяций хвоелистогрызущих насекомых. В соответствии с фенологической теорией динамики популяций (Мешкова, 2002), массовые размножения наиболее часто возникают в годы с синхронизацией периода питания фитофага с наличием подходящего корма, периода активности энтомофагов – с наличием восприимчивой стадии хозяина, неблагоприятных для фитофага погодных условий – с периодом его пребывания в наиболее защищенной стадии.

Определены для основных видов хвоелистогрызущих насекомых Украины экологические условия и участки, где имеется наибольшая вероятность развития очагов массового размножения, с учетом лесорастительных условий, возраста, полноты, состава насаждений, размещения участков в лесных массивах. Предложен алгоритм использования балльной оценки таких условий и баз данных лесоустройства для определения перечня и площади участков с наиболее вероятным возникновением вспышек. Приведен пример построения прогноза изменения угрозы вспышек хвоегрызущих насекомых с учетом изменения возрастного состава насаждений.

Проанализирована толерантность насаждений к повреждению рыжим сосновым пилильщиком (*Neodiprion sertifer* Geoffr.), ее зависимость от лесорастительных условий, возраста насаждений, начального санитарного состояния и уровня дефолиации в течение вспышки массового размножения. Приведен расчет вероятности изменения санитарного состояния деревьев сосны (*Pinus sylvestris* L.) в зависимости от их начального санитарного состояния и привлекательности лесных пород и участков для насекомых-фитофагов.

Ключевые слова: лесные насекомые, резистентность, толерантность,

ABSTRACT: Biotic factors of insect population dynamics include not only natural enemies but also interaction with host trees and site preferences.

R. Painter (1953) distinguished four types of interaction between phytophag and plant: *antixenosis*; *antibiosis*; *tolerance* and *avoidance* (pseudoresistance, phenological resistance).

The aim of this paper is to show the possibilities to obtain and use the knowledge about host tree and forest plot resistance and tolerance to insects for insect management.

Every phytophagous insect species is adopted to feed on foliage of one or several plant species, but the range of host plants depends on region, weather and phase of mass propagation of insects. Thus *Archips crataegana* Hb. prefers early form of oak (*Quercus robur*), during culmination of outbreak consumes almost all tree species, and at the end of outbreak preserves only on preferred tree species.

By data on food preferences of miner moths (*Phyllonorycter issikii* Kumata, 1963; *Cameraria ohridella* Deschka et Dimic, 1986; *Parectopa robiniella* Clemens, 1863; *Macrosaccus robiniella* Clemens, 1859) in the East Ukraine and in Transdniestria, conclusion is made about conditional resistance of these species to certain host plants.

Important role of phenological resistance in population dynamics of foliage browsing insects was proved. According to phenological theory of population dynamics (Meshkova, 2002), outbreaks are the most often in conditions of synchronization of feeding period for phytophags with the presence of suitable foliage, period of activity of entomophags – with the presence of the susceptible stage of host, and unfavorable for insects weather conditions must coincide with period of their stay in the most protected stage.

For the most spread foliage browsing insects, ecological conditions and plots with the highest probability of foci forming are determined taking into account forest site conditions, stand age, crown closure, the part of preferable tree species (pine for needle browsers and oak for leaves browsers), understory density, origin of the stand (vegetative or generative), plots location in the forest.

Algorhythm was developed for use of forest inventory data for determination of boundaries and area of potential foci of foliage browsing insects. Example of correction of prediction is shown taking into account the change of stand age.

Tolerance of stands to *Neodiprion sertifer* Geoffr., its dependence on forest site conditions, stand age, initial sanitary condition and defoliation during outbreak was analyzed. Probability of change of sanitary condition of pine (*Pinus sylvestris* L.) depending on initial sanitary condition was evaluated.

Key words: forest insects, tolerance, attractiveness of forest species and plots to phytophagous insects.

Introduction. Biotic factors of insect population dynamics include not only natural enemies but also interaction with host trees and site preferences.

Unlike pathogens, insects actively choose the trees for feeding or colonization. Their colonization is successful, if tree resistance is low. The last one can be passive or active and be conditioned by genetic or ecological causes.

R. Painter (1953) distinguished four types of interaction between phytophag and plant: *antixenosis* (non-preference) – rejection of resistant plants by insects for colonization, feeding, oviposition and development; *antibiosis* (true resistance) – direct influence of plant on insect viability after colonization the plant; *tolerance* **–** capability of plant to compensate the

damage, to minimize negative consequences for tree; *avoidance* (pseudoresistance, phenological resistance) – the host plant escapes infestation by not being at a susceptible stage when the insect pest population is at its peak. The last type prevents colonization and damage, but does not reveal itself under certain weather conditions or some management actions.

The aim of this paper is to show the possibilities to obtain and use the knowledge about host tree and forest plot resistance and tolerance to insects for insect management.

Materials and methods. Almost 40 year experience of author and publications on forest protection are the base of analysis.

Results. Every phytophagous insect species is adopted to feed on foliage of one or several plant species. Monophags have advantages in the rate of growth and productivity, but polyphags have a good chance to survive without the most preferable host plant.

At the same time, the range of host plants varies for different populations of the same pest or in different years.

Small-leaved linden (*Tilia cordata*) was infested in Kharkov region by Japanese linden midget moth (*Phyllonorycter issikii* Kumata, 1963). Limes *T. americana*, *T. tomentosa*, *T. europaea* and *T. sibirica* were relatively resistant to *Phyllonorycter issikii*, and *T. amurensis* and *T. japonica* were resistant in the years of our investigations (Meshkova et al., 2013).

In Kharkov region (East Ukraine) common horse chestnut (*Aesculus hippocastanum*) is damaged by the chestnut miner (*Cameraria ohridella* Deschka et Dimic, 1986). Horse chestnut *A. carnea* and *A. glabra* are relatively resistant to *C. ohridella*, and *A. parviflora* is absolutely resistant (Meshkova et al., 2013). In *A. carnea* in Kharkov larvae developed only to the IInd instar, and in Transdniestria (Antuhova, Meshkova, 2011) larvae survived to the IVth instar. Low vitality of horse chestnut miner on *A. carnea* can be explained by high density of its epidermis cells, which reveals in more dark coloring and rigidity in comparison with *A. hippocastanum*.

False acacia midget moth (*Parectopa robiniella* Clemens, 1863) and false acacia miner (*Macrosaccus robiniella* Clemens, 1859) are monophags in Transdniestria (Antuhova, Meshkova, 2011). In Kharkov region locusts *Robinia pseudoacacia* and *R. viscosa* were colonized by these moths, but *R. pseudoacacia* was more resistant to the both species (Meshkova et al., 2013).

Thus, recommendations on mass replacement of susceptible tree species with resistant ones are early, because adaptation of leafminers to them is possible in the next years.

Phenological resistance plays the most important role in population dynamics of forest pests. It can explain the differences in host species ranges in particular regions and years. Thus green oak leafroller, *Tortrix viridana* L. (Tortricidae) consumes mainly the foliage of early form of *Quercus robur* in the plain part of Ukraine (Meshkova, 2009). In Slovakia (Patocka et al., 1999) and Crimea (Ivashov, 2001) it consumes also foliage of *Q. petraea* and *Q. pubescens*. *Lymantria dispar* L. (Lymantriidae) consumes in Ural mainly *Betula sp.* and *Pinus sylvestris* (Ponomarev et al., 2012), in Volga region *Quercus robur* (Lyamtsev, 1986), and in Kherson region also *Robinia pseudoacacia* and *Populus sp*. (Kireeva, 1983).

Host preferences for phytophags can change during outbreak. Thus, in Kharkov region at the beginning of outbreak the larvae of *Archips crataegana* Hbn. (Tortricidae) consumed foliage of early form of oak (*Quercus robur*). Late

form of oak (*Quercus robur*) was damaged less than other tree species because of late flushing. In the year of outbreak culmination larvae consumed all tree species in the plot, and even defoliation of late oak came up to 40 – 50% (averagely 27.1%). The next year after culmination defoliation of all tree species decreased except oak of early form. During development of outbreak larvae consumed the foliage of more preferable and less preferable species. Feeding on *Acer sp*. and *Ulmus sp*. brought to low viability and fecundity of larvae in comparison with feeding on oak and *Tilia cordata*. Therefore to collapse of outbreak the most part of individuals was found in "preferable" trees, that is, their distribution became more aggregated (Meshkova, 2009).

Biochemical composition of foliage alters during vegetation period. Content of nitrogen and water decreases, the content of carbohydrates, tannins and phenolic compounds increases (Feeny, 1970; Haukioja, 2005). In accordance to it, every phytophag is adopted to the foliage of such composition which corresponds to certain period of foliage development. Thus, the larvae of *Tortrix viridana* and *Operophthera brumata* are adapted to feeding on young foliage. Larvae of *Lymantria dispar* in early instars consume young foliage, and from the 3rd instar consume old foliage. Larvae of *Euproctis chrysorrhoea*, on the contrary, in early instars consume old foliage (in August), and in old instars (after hibernation) consume young one. The larvae of *Notodonta anceps* Goeze (Notodontidae), *Calliteara* = *Dasychira pudibunda* L. (Notodontidae) and *Phalera bucephala* L. (Notodontidae) consume only old foliage (Meshkova, 2009).

Larvae of *Neodiprion sertifer* Geoffr. (Diprionidae) consume pine foliage of previous year (Kolomiets et al., 1972). Larvae of *Dendrolimus pini* L. (Lasiocampidae) consume the foliage of previous year, and young larvae (hatched in summer) eat the foliage of current year (Jerusalimov, 2004). Larvae of *Diprion pini* L. of spring generation consume the needles of previous year, and larvae of autumn generation eat the needles of current year (Meshkova, 2009).

According to phenological theory of population dynamics (Meshkova, 2002), the success of insect survival is provided only in conditions of synchronization of feeding period for phytophags with the presence of suitable foliage, period of activity of entomophags – with the presence of the susceptible stage of host, and unfavorable for insects weather conditions must coincide with period of their stay in the most protected stage.

The dates of larvae appearance for species hibernating as egg are influenced mainly by the course of air temperature after overcoming certain threshold. At the same time, the dates of spring development of the buds of tree species depend considerably on the dates of beginning of water suction by roots, and the last one begins only after soil thawing in the zone of the main mass of roots distribution.

Differences in the dates of buds development in the different points considerably depend on differences in the dates of soil thawing in different regions, relief, soil type, and its humidity before freezing.

At minimal time interval between budburst and larvae hatch, feeding of larvae by the youngest foliage with high protein and water contents as well as the most weakening of trees is provided, because the shoots are damaged in the buds before beginning of photosynthetic activity of leaves. Such situation is formed in such locations and in such years when budburst is detained in result of slow soil thawing and later beginning of water suction by roots.

Critical periods were determined in development of foliage browsing insects. Coincidence of the beginning of outbreaks for certain species and certain weather conditions in the critical periods of development were proved statistically.

On example of insects with different types of seasonal development it was shown, that population dynamics of many years (probability, severity, duration of outbreaks and intervals between them) is determined by peculiarities of seasonal development of insect in certain weather conditions of the region (Meshkova, 2009).

For planning of timetable and expenses on survey and control of foliage browsing insects, it is necessary to know the boundaries and area of potential foci of mass propagation, as well as to have a pictorial view of foci location in the stands, particularly thematic maps.

Variability of population dynamics in different forest site conditions was demonstrated on examples of *Archips crataegana*, *Neodiprion sertifer* and *Dendrolimus pini* (Meshkova, 2004). It was proved, that different viability and fertility of *Archips crataegana* in different forest plots is connected with tree composition, namely, the part of *Quercus robur* in the stand. Hatching began earlier in the most lighted plots and developed quicker. Undergrowth and blooming herbs were absent in such plots, and forest litter was rather thin there, which was unfavorable for feeding and hibernating of entomophags.

Outbreak of *Neodiprion sertifer* in pine stands of 35–40 years old was the most intensive and long in the dry bor (A_1). Viability of all stages of pine sawfly was the highest in this plot as compared with fresh bor (A_2) and fresh subour (B_2) (using typology of Alerseev-Pogrebnyak (Pogrebnyak, 1955)).

Population density of *N. sertifer* decreased mainly in result of virus epizooty, which developed more intensively in shaded plots with dense understory (B_2), whereas viral polyhedra on needle surface inactivated by ultraviolet radiation in well illuminated plots (without understory and deciduous plants) in A_1 and A_2.

Development of larvae of sawfly completed earlier in the most lighted and heated plots, so the first cocoons appeared in A_1, A_2 and B_2 on May16, 20 and 23, whereas swarming of the most spread entomophag (*Pleolophus* (=*Aptesis*) *basizonus* Grav.: Ichneumonidae) started since May 13, and the last had to pass maturating feeding before attacking just formed cocoons. It took more time in A_1, than in B_2, which reflected in low level of parasitism in the first forest conditions (Meshkova, 2004).

In the years of the highest density of sawfly population the interval between Icheumonid swarming and sawfly cocoons forming made up 10–11 days.

In the plot in B_2 the densest forest litter was favorable not only for hibernation of entomophags but also for their survival in hot summer. In the plot in A_1 and A_2 we found the cocoons of sawfly with dead larvae of parasitoid inside, which was connected with high soil temperature (up to 40 °C).

Population dynamic of *Dendrolimus pini* was also studied in A_1, A_2 and B_2 forest site conditions but in the stands of higher age (40 – 50 years old). The highest intensity and duration of outbreak were registered in A_1, and the lowest ones in B_2.

After culmination of outbreak of this pest the tachinid fly *Panseria (Ernestia) rudis* Fall. dominated among entomophags. It attacked the larvae of *Dendrolimus pini* in the 4^{th} instar and completed development in its pupae. This fly began swarming in 1994, 1995, 1996, 1997 and 1998 on June 3, May 13, 5,

13 and 12 respectively and spent several days on maturating feeding. So in 1994 the fly completed hibernation 10 days before pupation of *D. pini* and had to look for another host. The slower development of *D. pini* larvae in B_2 plot was the cause of their highest infestation by tachinid fly.

Analysis of population indices of foliage browsing insects allows pointing the ecological conditions and the plots with the highest threat of outbreak for these pests.

Forest site condition, stand age, crown closure, the part of preferable tree species (pine for needle browsers and oak for leaves browsers), understory density, origin of the stand (vegetative or generative), plots location in the forest were taken into account. The most of these characteristics are contained in the forest inventory databases and can be used for prediction the area of potential forest pest outbreaks as well as for thematic maps creation on the contours of stands, which are digitized for considerable part of forests of Ukraine (Meshkova, 2009).

For evaluation of plots preferences for foliage browsing insects, indices of electivity and selectivity were evaluated concerning forest site conditions, stand age and density, part of elected host plant, understory and herbage, origin of stands as well as plot location in the stand.

Electivity and selectivity indices, which show the extent of tree or plot "preference", were calculated taking into account "availability" and "utilization" of trees or plots.

"Availability" of trees of every species was evaluated as the ratio of sum of cross-sections on breast height for trees of given species and all tree species in the plot. "Availability" of plots with certain forest site conditions (age, density) was evaluated as the part of area for plots with such forest site conditions (age, density) from the whole area of surveyed plots.

"Utilization" of trees was evaluated for polyphags as the ratio of insect population density in the trees of given species to the average insect population density for all tree species or respective crown defoliation levels.

"Utilization" of some plots in the stands with given forest site conditions (age, density) was evaluated as the ratio of average insect population density in the plots with such forest site conditions (age, density) and average insect population density in all surveyed plots, or the ratio of number of plots with presence of given species in given forest site conditions (age, density) and the number of "nonzero" plots from all surveyed plots.

For complex evaluation of forest plots preferences for foliage browsing insects, electivity index was ranged by numerical score of 5 marks.

For example (Table 1), for pine defoliators according to forest site conditions mark 1 characterizes the plots, where insect can get occasionally, but do not survive. The stands in B_3, $C_1 – C_2$ (mark 2) are mainly mixed and only migratory foci can develop there. In the plots in A_3 and B_2 (mark 3) outbreaks begin later than in primary foci and fast extinguished. Intensity and duration of outbreaks of needle eating insects are higher in A_2 and B_1 (mark 4), and the highest in A_1 (mark 5).

The highest is the threat of outbreaks of oak defoliators in D_1 and D_2. It decreases in C_2 (mark 3). Mark 2 characterizes the plots in C_1, C_3, D_3, and in other forest site conditions outbreaks of oak defoliators are not registered at all.\

Plot preference by the age of the stands differs for oak and pine defoliators as well as for every species. The most of oak defoliators prefer the stands over 30 years old. *Neodiprion sertifer* colonizes the stands from the first

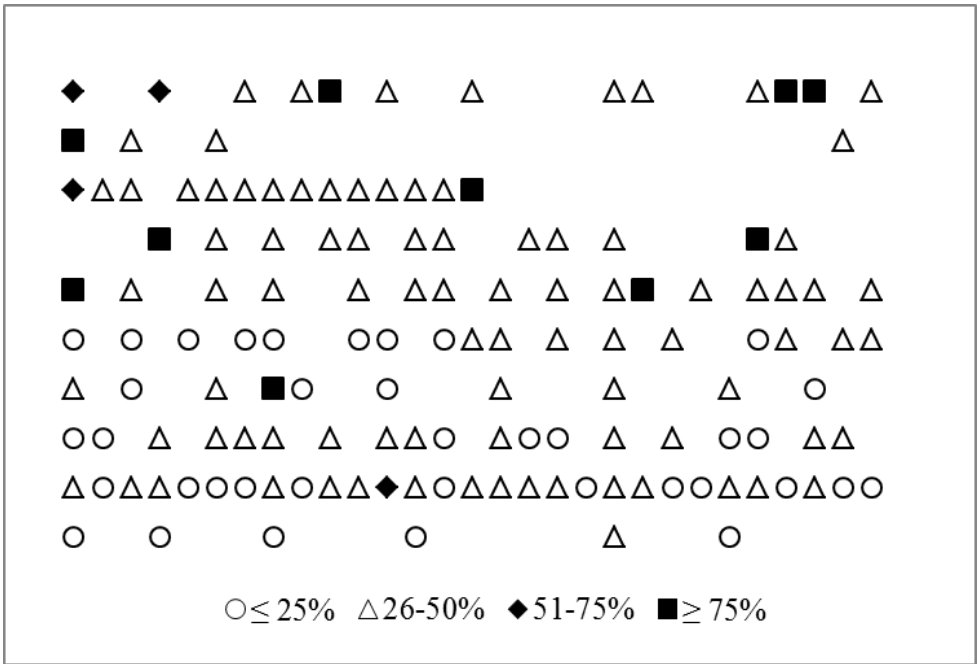
years of their growing, but develop the most dangerous outbreaks in the stands of 20–50 years old. *Dendrolimus pini* and *Bupalus piniarius* prefer older stands.

Table 1. **Rating of plot preference for foliage browsing insects depending on forest site conditions (Meshkova, 2009)**

Pests of pine		Pests of oak	
Mark	**Forest site conditions**	**Mark**	**Forest site conditions**
0	A_5, $B_4 – B_5$, $C_3 – C_5$, $D_1 – D_5$	0	$A_1 – A_5$, $B_4 – B_5$, $C_4 – C_5$, $D_4 – D_5$
1	A_4	1	$B_1 – B_3$
2	B_3, $C_1 – C_2$	2	C_1, C_3, D_3
3	A_3, B_2	3	C_2
4	A_2, B_1	4	D_2
5	A_1	5	D_1

The most of foliage browsing insects prefer the plots with good lightening and heating. Therefore the foci often form in the edges and in forest shelter belts. Since high biodiversity brings to higher resistance of stands, the probability of foci of all foliage browsing insects increases in monocultures. For pine defoliators the plots lose attractiveness when part of pine in the stand composition is less 70%, just when the foci of the most oak defoliators spread in the stands with even 60% oak.

All foliage browsing insects prefer the stands with low density of understory, as well as the plots adjacent to unclosed forest plantations and burnt area (Pic. 1). Such plots are well illuminated, quick heated and ecological conditions there are favorable for defoliators and unfavorable for hibernation and maturation feeding of parasitoids. Deciduous plants at the edges of pine stands are not favorable for pine defoliators, but favorable for their entomophags and prevent the spread of fire.



Pic. 1. Map of focus of *Neodiprion sertifer* (Lugansk region, 2008). Trees with the highest defoliation (black squares) are located mainly near gaps in the forest

Attractiveness the oak stands of vegetative origin is higher for defoliators as compared with the stands of seed origin.

For example, suitability of forest plot for *Tortrix viridana* by forest site conditions was evaluated as: mark 0 – A_1–A_5, B_4–B_5, C_4–C_5, D_4–D_5; mark 1 – B_1–B_3; mark 2 – D_3; mark 3 – C_1–C_3; mark 5 – D_1 – D_2. By stand age: mark 0 – up to 20 years old; mark 2 – 21–30 and over 80 years; mark 3 – 31–40 years; mark 5 – 41–80 years. By crown closure: mark 0 – not less than 80%; mark 4 – 70%; mark 5 – 60% and less. By the share of oak in the stand composition: mark 1 – 20% and less; mark 2 – 30–50%; mark 3 – 60%, mark 4 – 70–80%; mark 5 – 90% and more. By understory density: mark 1 – high; mark 2 – middle; mark 3 – low; mark 5 – absence. By forest origin: mark 3 – seed, mark 5 – vegetative. By location of sub-compartment in the forest: mark 1 – surrounded by forest, mark 2 – forest edge with shrubs, mark 4 –near vista or road, mark 5 – near unclosed plantations.

Forest sub-compartment suitability for *Operophthera brumata* by forest site conditions is: mark 0 – A_1–A_5, B_4–B_5, C_4–C_5, D_4–D_5; mark 1 – B_1–B_3; mark 2 – C_1, C_3, D_3; mark 3 – C_2; mark 5 – D_1 – D_2. By stand age: mark 0 – up to 20 years old; mark 1 – 21–30; mark 2 – 31–40 and over 80 years; mark 3 – 41–50 years; mark 4 – 51–60 71–80 years; mark 5 – 61–70 years. By crown closure: mark 0 – not less than 80%; mark 2 – 60–70%; mark 4 – 50%; mark 5 – 40% and less. By the share of oak in the stand composition: mark 2 – 20–60%; mark 3 – over 70%. By understory density: mark 0 – high; mark 2 – middle; mark 3 – low. By forest origin: mark 3 – seed, mark 5 – vegetative. By location of sub-compartment in the forest: mark 1 – surrounded by forest, mark 2 – near vista or road; 3 – forest edge with shrubs, mark 5 – near unclosed plantations.

Forest sub-compartment suitability for *Lymantria dispar* by forest site conditions is: mark 0 – A_1–A_5, B_4–B_5, C_4–C_5, D_4–D_5; mark 1 – B_1–B_3, D_3; mark 3 – C_1–C_3; mark 4 – D_2; mark 5 – D_1. By stand age: mark 0 – up to 20 years old; mark 1 – 21–30 and over 80 years old; mark 2 – 31–40 and 71–80 years; mark 4 – 41–70 years. By crown closure: mark 0 – not less than 80%; mark 3 – 70%; mark 4 – 50–60%; mark 5 – 40% and less. By the share of oak in the stand composition: mark 1 – 20%; mark 2 – 30–60%; mark 3 – 70–80%; mark 5 – 90% and more. By understory density: mark 1 – high; mark 2 – middle; mark 3 – low; mark 5 – absence. By forest origin: mark 1 – seed, mark 5 – vegetative. By location of sub-compartment in the forest: mark 1 – surrounded by forest, mark 2 – forest edge with shrubs, mark 3 – near vista or road, mark 4 – near unclosed plantations.

For complex evaluation of sub-compartments susceptibility for foliage browsing insects we calculated the sum of marks for every sub-compartment and then ranged it by 5-mark scale. The step was estimated as the difference between maximum and minimum dividing on 5.

For example, the maximally possible sum of marks by 7 site and stand characteristics is 35. Then the sub-compartments with sum up to 7 can be classified as the least susceptible to given insect (threat class I), the sub-compartments with the sums of marks 8–14, 15–21, 22–28 and 29–35 can be classified as II, III, IV and V class of threat.

For example, the plot in D_2 with oak stands (80% of oak) of vegetative origin of 50 years old, with crown closure 50%, low density of understory, neighboring unclosed plantations has the total mark 27 by susceptibility for gypsy moth and 28 for winter moth, which means IV class of threat of outbreak. Another plot is surrounded by forest, is located in C_3, the age of oak is 30 years, it is of vegetative origin, the part of oak is 40%, crown closure is 70%,

understory is middle. The total mark of susceptibility for gypsy moth is 17, and for winter moth it is 15, which means III class of threat of outbreak.

As forest inventory database contains the most of analyzed characteristics (except understory density and location in the forest massif), we have automatized calculations and completed additional fields in the database with the classes of threat for the main foliage browsing insects.

Algorhythm was developed for use of forest inventory data for determination of boundaries and area of potential foci of foliage browsing insects. Adequacy level for prediction dissemination of foliage browsing insects, comparing to ground survey, was determined accounting real and predicted numbers of plots preferred by certain insects as well as number of plots determined by the both methods.

Suggested approach was recommended for determination the list of plots, area and boundaries of potential foci of foliage browsing insects using forest inventory data and for creation of respective thematic maps. It gives the possibility to plan forest survey actions and to determine the plots where control measures are necessary.

Taking into account possible changes in stand structure, density, age and other characteristics, such evaluation must be repeated every 5-10 years.

Such evaluation was carried out for Doslidne Forestry of Steppe Branch (Kherson region) of Ukrainian Research Institute of Forestry & Forest Melioration taking into account the change of age of pine stands (Meshkova, Nazarenko, 2011). It was predicted, that the threat for the stands from *Neodiprion sertifer* and *Diprion pini* in 2015 would decrease as compared with 2005 from 3.9 to 3.7 and from 3.7 to 3.0 marks respectively. It would stay without change for *Dendrolimus pini* (4.4 marks), and increase for *Panolis flammea* from 3.1 to 3.3 marks. Respective change of foci area was evaluated (Table 2).

Table 2. **Distribution of area of pine stands by threat of outbreaks of pine defoliators evaluated by stand age for 2005 (numerator) and 2015 (denominator) (Doslidne Forestry)**

Species	Distribution of area, %					
	0 – threat is absent	1 – very low threat	2 – low threat	3 – middle threat	4 – high threat	5 – very high threat
Neodiprion sertifer	8.8 9.3	6.6 1.7	1.9 1.2	4.2 7.0	27.5 59.9	51.0 20.9
Diprion pini	0.0 3.5	19.6 14.5	8.0 5.8	1.3 48.8	20.1 6.4	51.0 20.9
Dendrolimus pini	0.0 3.5	4.2 3.5	0.0 0.0	6.1 4.7	35.5 17.4	54.2 70.9
Panolis flammea	0.0 3.5	12.9 12.8	12.7 6.4	22.0 7.6	52.3 69.8	0.0 0.0

Tolerance of stands to insect damage becomes apparent as the change in sanitary condition, growth intensity and mortality. These indices depend on the dates of defoliation, its level, weather, forest site conditions, stand structure and initial characteristics of sanitary condition and growth of trees. For example, height increment after defoliation can decrease 26–63% (Kulman, 1971), radial increment – 20–84%, and stem volume – 33–50% (Austara et al., 1987).

Deciduous plants (Rubtsov, Utkina, 2008) and *Larix sp*. (Pleshanov, 1982) at once after defoliation begin to restore foliage, if crown was damaged before completion of shoot growth or formation of tree ring. Therefore such species sustain repeated defoliation. Conifers usually sustain total defoliation caused by *Neodiprion sertifer*, which damages in spring the needles of previous year before completion the growth of current year needles. The trees sustain also defoliation caused by *Panolis flammea* up to 60%, which occurs in period of shoot growth. Needle damage by *Diprion pini* in August brings to greater losses of increment and often to tree mortality (Meshkova, 2013).

Sanitary condition of any living tree during lifespan can get better or worse. In the years with more favorable weather conditions and in the plots with favorable forest site the probability of improvement of sanitary condition increases. Such probability was evaluated for forest plots in Lugansk region in the foci of *Neodiprion pini* in the pine (*Pinus sylvestris* L.) stands. Forest site condition, age of stand, initially sanitary condition of tree, and defoliation level were taken into account (Meshkova, Kolenkina, 2014).

Table 3 shows an example of probabilities for pine trees to change sanitary condition for 2007–2012 in the focus of *Neodiprion pini* in dry bor (A_1).

We can see that 13.3 and 6.7% of trees (0.13 and 0.07) changed their sanitary condition from I (healthy) to V (recently died) and VI (died last year). Therefore predicted mortality of trees, which had I category in 2007, is calculated as product of initial part of trees of the I category (9.8%) and probability of their death (0.13+0.07=0.2) that is 9.8 * 0.2 = 1.96%. Similarly we calculate mortality of trees, which had II, III and IV sanitary condition in 2007. They are 5.23; 6.55 and 1.3%, and predicted mortality in the stand with such initial distribution of trees make up 1.96 + 5.23 + 6.55 + 1.3 = 15.04% (see Table 3).

Table 3. **Distribution of pine trees by sanitary condition in 2012 depending on their sanitary condition in 2007 (Lugansk region, dry bor A_2, 50 years old)**

Distribution of trees by sanitary condition classes, %							
2007*		2012 **					
		I	II	III	IV	V	VI
I	9.8	0.0	0.0	40.0	40.0	13.3	6.7
II	66.0	0.0	4.0	48.5	39.6	1.0	6.9
III	19.0	0.0	0.0	34.5	31.0	0.0	34.5
IV	1.3	0.0	0.0	0.0	0.0	0.0	100.0
V	0.7	0.0	0.0	0.0	0.0	0.0	100.0
VI	3.3	0.0	0.0	0.0	0.0	0.0	100.0
Total	100.0	0.0	2.6	42.5	35.9	2.0	17.0

Notes: * – part of trees from total in 2007; * – parts of trees from total in 2012.

Conclusions

Every phytophagous insect species is adopted to feed on foliage of one or several plant species, but the range of host plants depends on region, weather and phase of mass propagation of insects.

Important role of phenological resistance in population dynamics of foliage browsing insects was proved. Outbreaks are the most often in conditions of synchronization of feeding period for phytophags with the presence of suitable foliage, period of activity of entomophags – with the

presence of the susceptible stage of host, and unfavorable for insects weather conditions must coincide with period of their stay in the most protected stage.

Algorhythm was developed for use of forest inventory data for determination of boundaries and area of potential foci of foliage browsing insects. Tolerance of stands to foliage browsing insects depend on forest site conditions, stand age, initial sanitary condition and defoliation during outbreak.

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A METHOD FOR CLEANING OF POLYHEDRA OF NEODIPRION SERTIFER NUCLEOPOLYHEDROVIRUS FROM INSECT TISSUES

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СПОСОБ ОЧИСТКИ ПОЛИЭДРОВ ВИРУСА ЯДЕРНОГО ПОЛИЭДРОЗА РЫЖЕГО СОСНОВОГО ПИЛИЛЬЩИКА ОТ ТКАНЕЙ НАСЕКОМОГО

Описан разработанный авторами способ очистки вирусной биомассы рыжего соснового пилильщика от жира и других остатков тканей насекомых. Описана схема проведения процедур очистки и ее методология.

Ключевы слова: вирус ядерного полиэдроза рыжего соснового плиильщика, очистка.

Summary. Authors suggested method cleans viral polyhedra of *Neodiprion sertifer* nucleopolyhedrovirus from DNA, fat and other insect tissue residues what guarantees obtaining of virus preparation with purity necessary for conduction of molecular genetic studies. The developed scheme for purification of viral biomass is fast and reliable and can be used in the production of various baculovirus preparations and for laboratory studies.

Introduction

In the production process of baculovirus preparations, it is very important to purify virus polyhedra from various impurities, including DNA and fat from the insect host. Absence of DNA in baculovirus preparation is especially important for conduction of molecular genetic studies.

Practically all registered viral preparations used for plant protection are based on baculovirus polyhedra (Yang et al., 2012). Baculovirus polyhedra consist of virions immersed in the crystalline matrix of polyhedrin protein, which has high resistance to various environmental factors (Chiu et al., 2012).

Baculovirus preparations are used against a wide range of insect pests (Bakhvalov et al., 2001), including red pine sawfly *Neodiprion sertifer* (Hymenoptera, Diprionidae), which is one of the most common and dangerous pests of young pine plantations in Russia. Against its larvae VIRIN-Diprion based on *Neodiprion sertifer* nucleopolyhedrovirus was used in Russia earlier, but now it is not produced.

In the absence of a viral preparation, chemical insecticides are used to protect plants against this pest. Chemical insecticides lack selectivity in action (Aktar et al., 2009) and have a long period of half-life that can last for years depending on environmental conditions (temperature, pH, illumination, microbial composition of soil) (Krupke et al., 2012). In addition, artificial young pine plantations have a reduced level of biological diversity and correspondingly are less resistant to unfavorable conditions than natural pine

forests. This is why application of pesticides, and especially repeated treatments, is undesirable. Currently, a new baculovirus preparation against red pine sawfly larvae is being elaborated (Sergeeva, Dolmonego, 2012).

Neodiprion sertifer nucleopolyhedrovirus primarily infects intestine while fat body and other tissues of infected larvae stay intact and the virus-containing biomass derived from the dead larvae is largely enriched with insect tissue remains. Fat particles predominate among the remains of insect tissues and their resulting mass fraction can reach up to 50%. These fat particles constitute unnecessary part of baculovirus preparation and require removal.

In addition, high purity of virus material is important for identification of virus strains. Manufacturing scheme requires a high-efficiency reference strain of the virus and its use for each cycle of production process. Thus, a constant molecular genetic control and highly purified virus preparations are necessary for successful production process.

It is known how to purify polyhedra of the cottonworm virus, namely, distilled water is added to a biomass of perished caterpillars and then is homogenized. The resulting homogenate is filtered through two layers of capron fabric with 0.1 mm cells, then filtrate is centrifuged and the precipitate is lyophilized. As a filler, zeolite or silicic acid is used (Kolosov et al., 2009). However, this method of purification does not provide reliable removal of impurities of insect tissues from viral preparations. We did not find any scheme used for purification of baculovirus preparations that could solve this problem.

Thus, it is not impossible to clean polyhedra of *Neodiprion sertifer* nucleopolyhedrovirus from the remains of insect tissues with existing techniques. That is why we have developed a method for cleaning of the virus polyhedra of *Neodiprion sertifer* nucleopolyhedrovirus, providing a viral preparation with required level of purity for molecular genetic studies.

Materials and methods

The work was carried out with the viral biomass of intestinal nucleopolyhedrovirus isolated from dead larvae of red pine sawfly. Red sawfly larvae were collected in the natural centers of their mass propagation and under laboratory conditions viral biomass was obtained from them.

Results and discussion

To obtain viral preparations with the maximum degree of purity we developed a scheme for cleaning of viral polyhedra which includes homogenization of biomass of the dead larvae, centrifugation of homogenized biomass and purification of virus polyhedra from insect tissues, including DNA.

Initially, centrifugation is carried out at a speed of 13,000 rpm for 10-15 seconds in 1.5 ml eppendorf tube before the appearance of a white fraction of polyhedra over the sediment of insect tissues (centrifuge radius is 4 cm). The supernatant is removed to a new tube and a solution of 3-molar guanidine thiocyanate (100 µl of solution per 30-40 µl of precipitate) is added. Then centrifugation is carried out at a speed of 12,000 rpm for 1 minute. Distilled water is added and precipitate is gently resuspended with vortex mixing. After this step the polyhedra remain attached to the walls of the polypropylene test tube, and the remains of the insect tissues pass into an easily removable supernatant. Then the polyhedra are washed out again with water and the supernatant is removed.

Further, viral polyhedra are purified from DNA of the insect, which has a high affinity for them. To do this, TE buffer is added to the polyhedra sediment in a ratio of 1:50 (sediment:buffer) by volume and polyhedra are resuspended in buffer. (TE buffer is a solution consisting of a 1-molar Tris-HCl (pH 8.0) and

0.5-molar solution of ethylenediaminetetraacetic acid). The resulting solution at a temperature of +25 °C is held for 3 minutes and after is centrifuged at 5,000 rpm for 1 minute. To obtain a higher degree of purification of viral polyhedra, this procedure is performed two more times.

The absence of an insect DNA in the resulting material is checked by electrophoresis in a 0.9% agarose gel with ethidium bromide by a standard procedure used to detect DNA amplification products (Sambrook et al., 1989). If necessary, procedure for purification of the polyhedra from DNA is repeated once again.

Conclusion

The suggested method cleans viral polyhedra of *Neodiprion sertifer* nucleopolyhedrovirus from DNA, fat and other insect tissue residues what guarantees obtaining of virus preparation with purity necessary for conduction of molecular genetic studies. The developed scheme for purification of viral biomass is fast and reliable and can be used in the production of various baculovirus preparations and for laboratory studies.

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GENOMIC SEQUENCING AND ANALYSES OF HEARMNPV—A NEW MULTINUCLEOCAPSID NUCLEOPOLYHEDROVIRUS ISOLATED FROM *HELICOVERPA ARMIGERA*

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摘 要

背景:多粒包埋型棉铃虫核型多角体病毒（HearMNPV）是一种核型多角体病毒(NPV)能够感染棉铃虫（*Helicoverpa armigera*），该病毒粒子为多粒包埋型(在电子显微镜下可以看到)。HearMNPV病毒与单粒包埋型棉铃虫核型多角体病毒(HearSNPV)相比具有不同的寄主范围。为了更好地了解HearMNPV病毒,对HearMNPV基因组进行了测序和分析。

方法：应用电子显微镜对HearMNPV病毒的形态进行了观察。利用qPCR技术来确定和检测HearMNPV病毒感染棉铃虫体内复制动力学。根据“部分填满”方法构建了一个随机的HearMNPV基因组文库，对HearMNPV基因组的序列和结构进行了分析,并与其他杆状病毒基因序列数据进行了比较。

结果：实时qPCR检测表明，HearMNPV在棉铃虫感染过程中DNA的复制包括减少阶段,潜伏阶段,对数生长期和稳定期4个阶段。HearMNPV基因组由154196个碱基对组成，G+C含量为40.07%。HearMNPV基因组中有162个公认的开放阅读框，代表基因组的90.16%。剩下的9.84%构成了四个同源区域和其他非编码区域。HearMNPV的基因含量和基因排列与蓓带夜蛾NPV-B病毒(MacoNPV-B)非常相似， NPV-B(MacoNPV-B),但与单粒包埋型棉铃虫NPV不同。比较了HearMNPV和MacoNPV-B基因组后推测HearMNPV缺失了包含在5个开放阅读框中5.4Kb片段。另外,HearMNPV*orf66*、*bro*和*hrs*基因与MacoNPV-B基因组的相应部位不同。

结论: HearMNPV病毒可以在棉铃虫体内和体外进行复制，是一种新的有别于HearSNPV的分离株。HearMNPV病毒与MacoNPV-B病毒非常近似，但又有独特的基因结构、含量和机制。

关键词：杆状病毒，棉铃虫，多粒包埋型核型多角体病毒，基因序列比较

Key words: Baculovirus, *Helicoverpa armigera*, Multinucleocapsid nucleopolyhedrovirus, Genome sequence comparison

НОВЫЙ МУЛЬТИНУКЛЕКАПСИД ВИРУСА ЯДЕРНОГО ПОЛИЭДРОЗА ГУСЕНИЦ HELICOVERPA ARMIGERA

Изучен сложный многоклеточный вирус ядерного полиэдроза *Helicoverpa armigera* (HearMNPV). ПЦР-анализ в реальном времени показал, что репликация ДНК HearMNPV в процессе развития болезни у

гусениц включала четыре стадии: активизация вируса, скрытый период развития болезни, быстрое развитие болезни и стабилизация. HearMNPV-геном состоит из 154196 пар оснований, содержание G + C 40,07%. В геноме HearMNPV имеется 162 признанных открытых кадра считывания, что составляет 90,16% генома. Остальные 9,84% составляют четыре гомологичных региона и другие некодирующие области. Сравнивая геномы HearMNPV и MacoNPV-B, мы предполагаем, что HearMNPV не содержит фрагмента 5,4 кб, содержащегося в пяти открытых кадрах считывания. Кроме того, гены HearMNPVorf66, bro и hrs отличаются от соответствующих сайтов генома MacoNPV-B.

Ключевые слова: вирус ядерного полиэдроза Helicoverpa armigera, ПЦР-анализ, геном.

Background

Members of the family Baculoviridae are rod-shaped viruses with circular, covalently closed, double-stranded DNA genomes [1]. This family includes four genera: *Alphabaculovirus* (lepidopteran-specific nucleopolyhedroviruses(NPVs)), *Betabaculovirus* (lepidopteran-specific granuloviruses), *Gammabaculovirus* (hymenopteran-specific NPVs) and *Deltabaculovirus* (dipteran-specific NPVs) [2]. To date, 54 baculovirus genomes have been sequenced (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi? taxid=10442), including 37 from *Alphabaculovirus*, 13 from *Betabaculovirus*, three from *Gammabaculovirus* and one from *Deltabaculovirus*. Nucleopolyhedrovirus (NPV) and granulovirus (GV) are distinguished from each other by their occlusion body morphology. The NPVs produce large, polyhedron-shaped occlusion bodies, called polyhedra, which contain many virions, whereas the GVs have smaller occlusion bodies, called granules, which normally contain a single virion. The NPVs are further designated as single-nucleocapsid (S) or multinucleocapsid (M), depending on the potential number of nucleocapsids packaged in an envelope of the virion.

The cotton bollworm, *H. armigera*, is a serious pest that causes economic losses to over 60 vegetable and field crops throughout the world [3]. *H. armigera* larvae are significantly resistant to chemical insecticides; therefore, baculovirus pesticides have been recognized as one of the most promising agents to control such pests [4]. HzSNPV was registered as one of the first commercial baculovirus pesticides (Virion-H, Biocontrol-VHZ, Elcar) in the 1970s, and has been used extensively to control the cotton bollworm in the USA [5]. HearSNPV was also the first commercial baculovirus pesticide used to control *H. armigera* in China, and has been extensively used for the control of the pests in vegetable crops [6].

The DNA genomes of HearSNPV-G4 [7], HearSNPVC1 [8], HearNPV-NNg1 [9], and HzSNPV [10] have been sequenced. Among them, the HearSNPV-G4 and HearSNPV-C1 were isolated from China, HearNPVNNg1was isolated from Kenya, and HzSNPV was isolated from the United States. Comparative genomic analyses showed that overall gene content and arrangement in these four viruses were highly conserved, and they are considered variants of the same NPV species [9]. In addition, the nucleotide sequence of the HearGV DNA genome was reported [11]. Multinucleocapsid NPVs isolated from *H. armigera*(HearMNPV) producing ODV virions with multiple nucleocapsids per envelope have been identified [12,13]. The genes of other 18 HearMNPV isolates from *H. armigera* which included *lef-8, lef-9*, polyhedrin have been reported [14].

In this study, a new nucleopolyhedrovirus isolated from *H. armigera* was observed by electron microscope (EM), suggesting it was multinucleocapsid NPV. Experimental infection of insect larvae indicated that host range of HearMNPV was different from that of HearSNPV and that the cytopathological effect of HearMNPV differed from that of HearSNPV. This report describes the sequence and organization of the HearMNPV genome and compares it with sequence data from other baculoviruses, such as HearSNPV and MacoNPV-B.

Methods

Viruses and insects

HearMNPV was originally isolated from a naturally infected *H. armigera* in the Shanghai city, China in the 1970s. The virus was propagated in laboratory stocks of healthy third instar *H. armigera* larvae by *per os* infection. A laboratory stock of eastern armyworm, cotton leaf worm and beet armyworm were reared at 26°C with a 16:8 h light:dark cycle on a semi-synthetic diet.

Virus purification, DNA extraction, and construction of genomic DNA libraries

To generate a large number of polyhedra, healthy third instar *H. armigera* larvae were inoculated and the hemolymph was collected from the *H. armigera* larvae were collected on ice and centrifuged for 10 min at 4 °C. The precipitate was washed several times with distilled water and re-suspended in 0.1% SDS for 30 min at room temperature. After centrifugation, the clean polyhedra were re-suspended in 200 μl TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) [15].

The genomic DNA of HearMNPV was purified according to the following protocol: about 5×10^8 polyhedra were dissolved in 0.1 M Na_2CO_3, 0.15 M NaCl, pH10.4 on ice for 10 minutes, SDS was then added to a final concentration of 0.5%, and the solution was kept on the ice for another 10 minutes. The genomic DNA was extracted twice in an equal volume phenol (pH 8.0) and once in chloroform. The DNA was precipitated with two volumes ethanol, washed with 70% ethanol, and dissolved in 0.1 × TE buffer (pH8.0) [16]. The quantity and quality of the isolated DNA were determined by spectrophotometrically and by electrophoresis on 0.7% agarose.

A random genomic library of HearMNPV was constructed according to the "partial filling-in" method and contained 2.0 to 5.0 kbp fragment in vector pUC19 [15,16] DNA fragments for sequencing were prepared from 527 recombinant plasmids. The recombinant plasmids were sequenced with plasmid specific primers and 'primer nesting' from both strands, using BigDye Terminator v3.1 (ABI) on a 3130XL Genetic analyzer (ABI). The combined sequence was generated from these clones represented a six-fold genomic coverage. The gaps were filled by PCR.

The insect cell lines and infection

The Hz-AM1 cell line and HaBacHZ8-GFP were gifts from Dr. Fei Deng of Wuhan Institute of Virology, Chinese Academy of Sciences. HaBacHZ8 is a bacmid of HearNPV that lacks the *polyhedrin* gene. An enhanced GFP gene was introduced to HaBacHZ8 by using the HearSNPV bac-to-bac system [17,18] and this generated the bacmid HaBacHZ8–GFP [19]. The QB-Ha-E-5 cell line, which was a gift from Dr. Guiling Zheng of Shandong Agricultural University, was established from the embryonic tissue of *H. armigera* (Lepidoptera: Noctuidae). The cell line had been subcultured over 60 passages in TNM-FH medium supplemented with 10% fetal bovine serum. The cell line could be infected by *H. arigera* single nucleopolyhedrovirus (HaSNPV) [20]. The HzAM1 cells and QB-Ha-E-5 cells were cultured at 27°C in TNM-FH insect medium (Sigma, USA) supplemented with 10% (v/v) heat-inactivated fetal

bovine serum (Gibco-BRL, Gaithersburg, USA). Hz-AM1cells and QBHa-E-5 cells were infected with HearMNPV at a multiplicity of infection (MOI) of 5. For coinfection, QB-Ha-E-5 cells were infected simultaneously with HearMNPV and HaBacHZ8-GFP at an MOI of 5 for each virus. The cells were examined using Nikon-Ts100 and Leica TCS SP5 II microscopes.

Scanning electron microscopy

Polyhedra were fixed in 2.5% glutaraldehyde at 4°C for 2 h. The fixed sample was dehydrated through a serial ethanol gradient, and then embedded in Epon-Araldite resin. A diamond knife was used to cut ultrathin sections on a Reichert OMU3 Ultramicrotome. The sections were stained with 2% aqueous uranyl acetate, followed by lead citrate. Micrographs of the Polyhedra were taken with a Hitachi S3400N transmission electron microscope at 80 kV.

Transmission electron microscopy

Polyhedra were fixed in 2.5% glutaraldehyde in 0.05 cacodylate buffer at 4°C for 2 h and post-fixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature. 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 JEM-1230 transmission electron microscope (TEM) at an accelerating voltage of 80 kV.

Quantitative PCR (qPCR)

Third-instar larvae were starved for 12 h at 26°C before being inoculated, and *H. armigera* test larvae were allowed to ingest a diet soaked in a 10 μl drop, containing an estimated 10^7 OBs. Control larvae ingested a diet soaked in a 10 μl drop with no OBs. The diet soaked OBs or ddH_2O were replaced by fresh diet with no OBs after 2 hours adsorption period at 26°C. Time zero of the infection was defined as the time when the diet soaked OBs or ddH_2O was removed from the culture boxes. Larvae used in this experiment were sacrificed at various time points ranging from 4 to 96 h postinoculation (p.i.). A powder was prepared from ten larvae using a mortar and pestle after each collection under liquid nitrogen. $0.1MNa_2CO_3$, 0.15 M NaCl, 1 %NP -40 was added to the powders to a total volume of 700 μl. Total DNA was then extracted by the addition of an equal volume of phenol (pH8.0) (twice) and chloroform (once). The DNA was precipitated with two volumes of ethanol, washed with 70% ethanol, and dissolved in ddH_2O. The quantity and quality of the isolated DNAs were determined spectrophotometrically.

HearMNPV DNA copy number was determined by real-time qPCR with primers specific to the rr2b gene. The viral copy number was then normalized against host-genome copy number by qPCR with primers specific to the host actin gene [21]. The rr2b and actin genes were amplified by PCR and cloned into pGEM-T. Recombinant plasmid DNA concentrations were quantified using a spectrophotometer and dilution standards were generated. For each standard dilution, three independent qPCRs were performed using *rr2b* or actin specific primers, and standard curves were generated. For each larval DNA extract, three independent qPCRs were performed using *rr2b* and actin specific primers. The mean of the HearMNPV DNA copy numbers were determined and the number of *rr2b* amplicons was normalized against the number of host actin genes to derive the mean number of viral copies per mean host actin gene copy number. The specific primers were as follows:

actin-F 5' CTCTTCCAGCCCTCATTCTTG 3 '

actin -R 5' TTCTGCATACGGTCAGCGATA 3 '

rr2b-F 5' AGCAACAAGACTTAATACTCAACGC 3 '

rr2b-R 5' AATATGGCTGCAAAGCTACCG 3 '

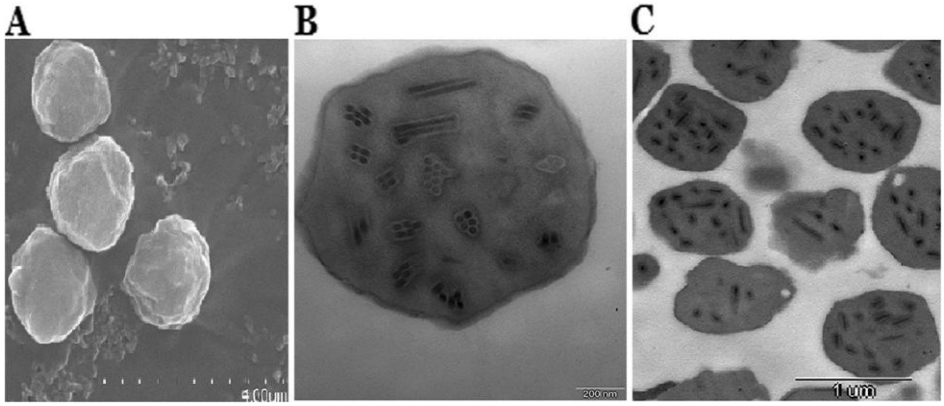
DNA sequence analysis

Restriction fragments from recombinant plasmids were sequenced and assembled into contigs using SeqMan5.0 from the DNASTAR software package. PCR was used to generate gap-spanning fragments and low quality data regions after preliminary assembly. Open reading frames (ORFs) were identified using ORF Finder http://www. ncbi.nlm.nih.gov/gorf/gorf.html [22]. The criterion for defining an ORF was a size of at least 150 nt (50 aa) with minimal overlap. Promoter motifs present upstream of the putative ORFs were screened as described previously [23]. Homology searches were done through the National Centre for Biotechnology Information (NCBI) website using BLAST [24]. Multiple alignments and percentage identities were performed using the Clustal W. The Tandem Repeats Finder http://tandem.bu.edu/ trf/trf.html was used to locate and analyze the homologous regions (*hrs*) [25]. GeneParityPlot analysis was performed as described previously [26]. A phylogenetic tree was inferred from amino acid sequences by NJ and MP analyses using MEGA, version 5.0 [27]. Bootstrap analyses were performed to evaluate the robustness of the phylogenies using 1000 replicates for both NJ and MP analyses.

Results and discussion

Electron microscopy observation

Scanning electron microscopy revealed that the purified occlusion bodies (OBs) of NPV originating from infected cotton bollworm have irregular shapes, with diameters of about 2 ± 0.3 μm (Pic. 1A). Transmission electron microscopy showed multiple rod-shaped nucleocapsids of about 230 nm in length and 50 nm in width embedded in each OB, with multiple nucleocapsids packaged within the envelope of the virion (Pic. 1B). These results indicated that the virus is a typical multinucleocapsid NPV. However, transmission electron microscopy indicated that HearSNPV have single nucleocapsids packaged in their virion (Pic. 1C). Therefore, the isolate was termed *H. armigera* multinucleocapsid nucleopolyh edrovirus (HearMNPV).

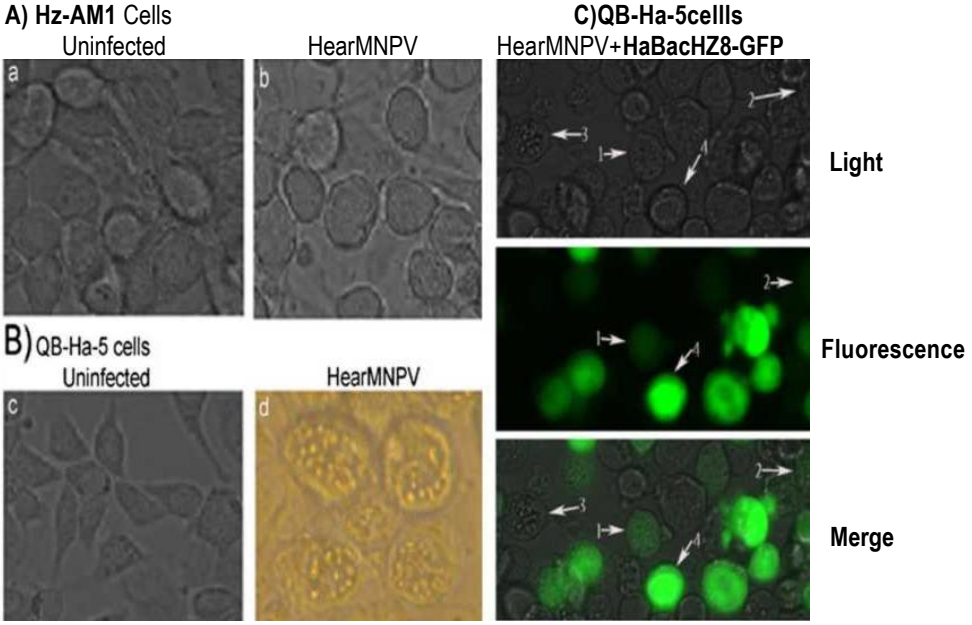


Pic. 1. Electron micrographs of polyhedra from HearMNPV and HearSNPV. A. Scanning electron micrograph of HearMNPV (12,000x) B.Transmission electron micrograph of HearMNPV (80, OOO*) C Transmission electron micrograph ofHearSNPV (20,000*).

HearMNPV infected insect larvae and cells

Experimental infection of insect larvae showed that HearMNPV can infect the eastern armyworm (*Pseudaletia separate*), but cannot infect either the cotton leaf worm (*Spodoptera litura*) or the beet armyworm (*Spodoptera exigua*). By contrast, HearSNPV cannot infect *P. separate*. These results indicate that the host range of HearMNPV differs from that of HearSNPV. Moreover,

HearMNPV-infected Hz-AM1 cells produced no polyhedra and showed no typical cytopathic effects (CPE), even at 96 h post-infection (pi) (Pic. 2A). However, HearMNPV-infected QB-Ha-E-5 cells produced polyhedra (Pic. 2B). It was previously reported that Hz-AM1 cells were permissive to HearSNPV-G4 [8,17,18]. When QBHa-E-5 cells were infected by HaBacHZ8-GFP [18,19] , which was constructed from HearSNPV, green fluorescence was observed under fluorescence microscopy (Pic. 2C). These results indicate that the host range and cells infected by HearMNPV differ from those infected by HearSNPV.



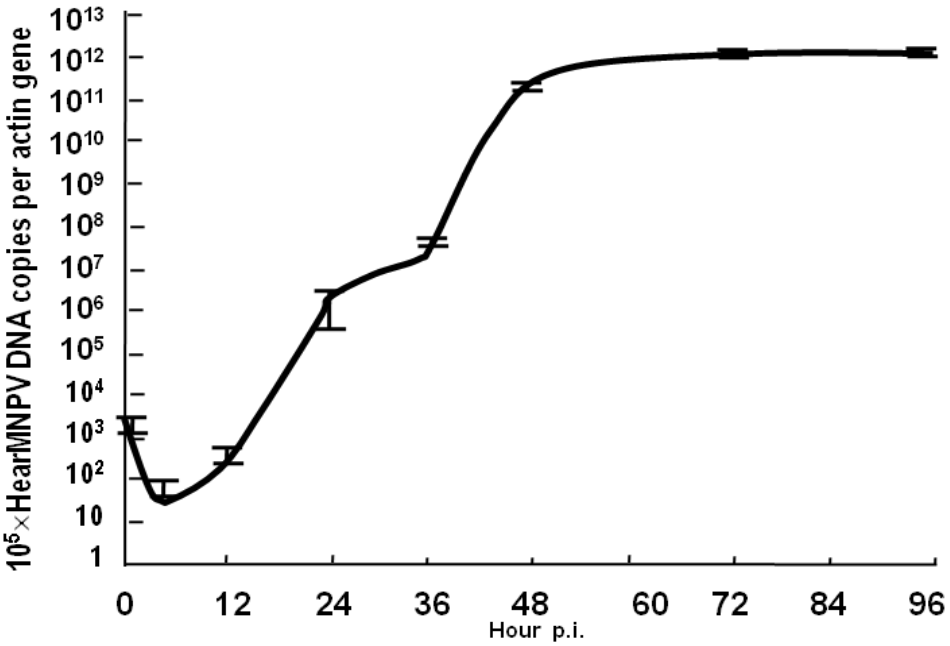
Pic. 2. Cytopathic effects in infected cells. Panel A: Hz-AMtellsuninfected (A) and infected with HearMNPV (MOI5)at 72 hpi (B) Panel QB-Ha-E-5 cells uninfected (Q and infected with HearMNPV (MOI:5) at 72hpi (D) Panel C QB-Ha-E-5cells co-infected with HearMNPV and HaBacHZ8-GFP (MOI5) at72hpi.Cells wereviewed using confocal laser fluorescence microscopy. Arrowsl and 2 indicate polyhedra-containing fluorescent cells, arrow3 indicates polyhedra-containing cells, andarrow 4 indicates fluorescent cells. Magnification: *400.

Coinfection of QB-Ha-E-5 cells with HaBacHZ8-GFP and HearMNPV showed that certain cells possessed green fluorescence (under fluorescence microscopy), some cells produced polyhedra, and some cells possessed both green fluorescence and polyhedra (Pic. 2C). The results indicate in cells coinfected with two distinct viruses, the viruses are able to coexist, replicate, and package themselves independently. *Cydia pomonella* granulovirus (CpGV) is one of the most successful commercial baculovirus insecticides; however, resistance of the codling moth (*C. pomonella*) to commercially applied CpGV in orchards located in Germany and France has occurred [28]. Therefore, alternating virus treatment or using a mix of HearMNPV and HearSNPV could delay the development of resistance in *H. armigera*, helping to improve both the prevention and control of *H. armigera* in the field.

HearMNPV virus DNA replication in vivo

HearMNPV is a potentially new isolate infectious for *H. armigera* based on the analysis of host range and morphology. Thus qPCR was used to

determine the replication efficiency of HearMNPV infectious for *H. armigera* in vivo. Pic. 3 shows the quantity of HearMNPV viral DNA in infected larvae, in the decreasing phase (0–4 hr), latent phase (4–12 h), exponential phase (12–48 h), and stationary phase (48–96 h). Initially, the number of viral DNA (vDNA) copies appeared to decrease between 0 h post infection (p.i.) and 4 h p.i. before increasing by 6.97 times between 4 h p.i. and 12 h p.i. The number of vDNA dramatically increased between 12 h p.i. and 48 h p.i., from $452/10^5$ actin to $2.02 \times 10^{11}/10^5$ actin, an increase of 4.46×10^8 fold. These results indicated that the viral DNA replicated about 29 times, taking about 1.24 h to generate another vDNA copy. This trend continued into the stationary phase, to a lesser degree: vDNA increased 4.82 times between 48 h p.i. and 96 h p.i, and there were about1.17×10^{12} copies per 10^5 actin at 96 h. These results suggested that *H. armigera* could be infected by HearMNPV efficiently and 15,170 bp, which represented 90.16% and 9.84% of the genome, respectively. The four hrs were distributed along the genome, with sizes ranging from 724 to 1,766 bp, and their total sequence was 4,749 bp, and the replication kinetics conformed what has previously been described for other baculoviruses [29].



Pic. 3. Quantification of HearMNPV DNA copy number in infected H. armigera larvae. qPCR was used to determine Ihe number of rr2b gene copies relative to Ihe actin gene at various times lb Howling infection. The plotted points indicate Ihe averages of the number rr2b gene copies relative to actin gene (performed in triplicate). Error bars represent standard deviations.

Nucleotide sequence of the HearMNPV genome

The HearMNPV genome consists of 154,196 bp (GenBank accession no. NC_011615), which is similar to the genomes of MacoNPV-A (155,060 bp) and MacoNPV-B (158,482 bp). The HearMNPV genome has a G + C content of 40.07%, which is within the 58% (LdMNPV) and 32.7% (ChocGV) range for baculovirus genomes, and is similar to MacoNPV-B (40%), AcMNPV (40.7%) , BmNPV (40.4%), and EppoNPV (40.7%). According to the adopted convention,

the adenine residue at the translation initiation codon of the *polyhedrin* gene represented the zero point on the HearMNPV physical map, and the polyhedrin gene was designated as ORF 1 (Table 1). A total of 162 putative ORFs and four homologous regions (*hrs*) were detected in the HearMNPV genome, using computer-assisted analysis to select ORFs starting from a methionine-initiated codon (ATG) and including at least 50 amino acids (aa) and having a minimal overlap with other ORFs [30,31]. All 162 ORFs are shown in Table 1 by location, orientation, size, and potential baculovirus homologs.

Table 1. **List of ORFs in HearMNPV and their Homologous ORFs in the MacoNPV-B, MacoNPV-A, HearSNPV(G4), AcMNPV, AgSeNPV and HearGV**

ORF Name Position[a] Prm[b] length Homologous ORFs[ORF number,amino acid identity(%)]

						MacoNPV-B		MacoNPV-A		HearSNPV(G4)		AcMNPV		AgSeNPV		HearGV	
1	polh	1	>	741	L	246	1	100	1	98.0	1	87.4	8	89.3	1	89.8	1	55.1
2	1629	790	<	2358	L	522	2	95.8	2	72.3	2	26.6	9	27.9	2	33.0	2	16.7
3	pk1	2357	>	3175		272	3	99.6	3	87.5	3	48.5	10	38.7	3	58.6	3	34.6
4	hoar	3250	<	5478	E	742	4	97.1	4	74.3	4	20.2			4	27.7		
5		5967	>	6533	E	188	5	96.8	5	67.2								
6	odv-e56	6626	>	7747	L	373	6	100	6	89.3	15	52.6	148	49.2	6	59.5	14	42.1
7	me53	7892	<	8956		354	7	99.7	7	86.2	16	30.9	139	24.6	7	47.4	178	22.1
8	F protein	9563	>	11599	L	678	8	99.9	9	90.9	133	36.9	23	22.1	8	44.4	26	29.8
9		11719	<	12678	E	319	9	99.1	10	91.2					9	46.1		
10	gp16	12729	<	13016	L	95	10	100	11	97.9	119	30.8	130	35.5	10	65.6		
11	p24	13029	<	13718	L	229	11	100	12	92.5	118	50.4	129	37.4	11	63.8	78	25.7
12		13784	>	14095	L	103	12	100	13	91.3	117a	34.6			12	44.4		
13	lef2	14049	>	14696		215	13	99.1	14	82.0	117	47.9	6	40.6	13	56.6	33	26.8
14	xe	14792	>	15175	E	127	14	99.2	15	85.5							21	22.0
	hr1	15251		16435														
15	endonuclease	16436	<	16732	L	98	15	99.0	17	93.4			79	44.7			69	40.5
16		16798	>	17397		199	16	98.5	18	87.9			70	23.9	25	27.0		
17		17550	>	18260	E,L	236	117	41.2	19	80.6			151	33.0	103	38.9		
18	chitinase	18318	<	20006	L	562	19	99.3	22	97.0	41	63.3	126	67.2	23	82.6	105	60.3
19	bro-a	20197	>	21192		331	20	83	24	77.7	60	53.9	2	23.3	50	33.2	101	64.3
20		21257	>	21682	E	141	21	99.3	25	86.5							131	38.9
21		21786	>	22595	E	269	22	98.1	26	78.1							52	40.8
22		22706	<	23347		213	23	95.8	27	91.1	57	25.8			49	21.4	81	34.9
23		23525	>	23839	L	104	24	99.0	29	64.4								
24		23960	<	24595	E	211	25	97.6	30	69.2								
25	hel2	25012	<	26379	E	455	26	98.9									147	57.7
26	he65	26512	<	28266		584	27	98.5	32	91.7	61	28.6	105	37.1	20	53.3	62	53.4
27	cathepsin	28331	>	29356		341	28	100	33	97.1	56	46.7	127	56.9	19	81.3		
28		29353	<	29700	L	115	29	100	34	90.6	125	29.0			18	72.0		
29	lef1	29728	>	30375		215	30	99.1	35	95.8	124	48.2	14	40.0	17	64.2	80	36.6
30	38.7 k	30375	>	31424	L	349	31	99.7	36	92.6	123	34.1	13	29.5	16	60.3	102	23.4
31	gp37	31476	>	32264	L	262	32	99.6	37	95.0	58	62.8	64	58.6	26	72.4	109	43.1
32	ptp2	32221	<	32760	L	179	33	98.9	38	92.7					27	51.0		
33	egt	32828	>	34414	E	528	34	99.4	39	94.4	126	50.7	15	48.0	28	73.1		

34		34583	>	35119	E	178	35	98.9	40	94.9	127	22.3			29	53.3		
35		35119	>	35766		215	36	98.1	41	90.7	128	28.1	17	30.4	30	45.9		
36		35802	<	38357		851	37	99.4	42	88.5	129	26.5			31	45.4		
37	chtB2	38415	>	38855	L	146	38	98.6	43	64.6	83	31.7	146	43.8	148	34.0	107	46.2
38		38886	>	39413	L	175	39	99.4	44	82.8			4	26.7	32	36.8		
39	pkip	39434	>	39943	L	169	40	100	45	91.6	130	36.2	24	23.5	33	55.6		
40		39965	<	40306		113	41	100	46	96.5					34	52.7		
41	arif1	40312	<	41184		290	42	98.6	47	92.0	131	29.2	21	22.7	35	43.3		
42	pif2	40940	>	42199		419	43	99.3	48	95.0	132	69.0	22	59.5	36	74.6	42	51.1
43	pif1	42214	>	43803		529	44	99.6	49	93.6	111	43.0	119	48.6	37	61.2	82	36.2
44		43800	>	44045		81	45	100	50	96.3	112	32.9			38	44.3		
45	fgf	44080	<	45174		364	46	99.2	51	72.9	113	32.0	32	27.5	39	41.7	176	34.0
46		45210	>	46115		301	47	99.7	53	89.5					40	45.7		
47	alk-exo	46157	<	47344	L	395	48	99.8	54	90.1	114	41.4	133	38.8	41	48.6	146	39.4
48		47582	<	47920	L	112	49	100	55	93.6	115	27.0	19	26.7	44	65.1		
49		47919	>	49082	L	387	50	99.7	56	94.6			18	25.8	45	62.3		
50		49121	<	49522		133	51	99.3	57	94.7	122	22.5	132	21.9	46	50.0		
51	rr2	49594	>	50535		313	52	99.7	58	94.3					47	72.2		
52		50544	<	51593		349	53	100	59	73.1					103	55.3		
53	calyx/pep	51620	<	52597		325	61	100	60	97.5	120	41.6	131	32.3	49	65.7	18	21.0
54		52871	<	53209		112	62	100	62	84.1	110	40.2	117	34.2	51	66.0		
55		53161	<	53523	E	120	63	100	63	90.8	109	35.8			52	53.2		
56		53687	<	54301	E,L	204	64	98.5	65	86.8								
57	sod	54366	<	54821		151	65	100	66	96.7	106	75.3	31	73.2	54	85.2	63	53.7
58		54878	>	55243		121	66	100	67	86.8					55	47.3		
59	pif3	55269	>	55880	L	203	67	98.0	68	92.6	98	51.8	115	47.1	56	70.0	30	44.5
60		55846	>	56316		156	68	98.1	69	87.7	99	22.7			57	57.5		
61	pagr	56365	>	57819	L	484	69	99.4	70	87.4	100	23.5			58	38.0		
62		57842	>	58483	L	213	70	100	71	96.8	101	57.1	106	53.2	59	78.2	45	44.1
63	nrk1	58518	<	59627	E	369	71	99.4	72	95.0			33	30.1	60	58.4		
	hr2	59758		61523														
64		61569	>	62045	L	158	72	93.7	73	87.3			4	25.6	32	20.2		
65	dutpase	62105	>	62449	E	114	73	91.2	74	92.2					62	66.7		
66		62531	>	64309	E,L	592	18	99									53	51.0
67	bro-b	64565	>	65572		335	74	77.5	75	79.6	60	47.5	2	23.0	50	32.0	101	54.8
68	p13	65621	>	66457	L	278	75	99.6	76	95.3	97	58.4			64	68.5	40	47.4
69	sprT	66512	>	67036		174	76	98.9	77	89.7							21	30.7
70	odv-e66a	67148	>	69166	E,L	672	77	99.7	78	97.2	96	58.1	46	39.7	125	35.9	150	56.3
71		69163	<	69474	L	103	78	99.0	79	96.1	95	44.7	108	32.8	65	59.6		
72	odv-ec43	69484	<	70554	L	356	79	100	80	95.2	94	50.4	109	41.6	66	77.3	48	31.9
73		70538	<	70717		59	80	100	81	98.3	93	50.0	110	36.0	67	73.7	46	50.0
74	vp80	70714	<	72360		548	81	99.3	82	83.1	92	24.0	104	22.9	68	37.2		
75	p45	72388	>	73521	E,L	377	82	99.7	83	97.9	91	58.7	103	51.1	69	80.7	90	39.5
76	p12	73508	>	73816	L	102	83	100	84	89.2	90	42.1	102	32.6	70	51.9		
77	p40	73842	>	74936	L	364	84	100	85	92.6	89	53.1	101	43.4	71	69.6	92	21.6
78	p6.9	74995	>	75228	L	77	85	100	86	83.3	88	58.9	100	62.0	72	64.2	93	16.7
79	lef5	75225	<	76046		273	86	99.6	87	96.3	87	50.3	99	57.4	73	77.6	94	43.0
80	38 k	75945	>	76847	L	300	87	99.7	88	96.0	86	53.7	98	44.6	74	73.0	95	40.5
81	vef	76886	>	79432	L	848	88	99.3	89	81.3					76	39.5	151	28.4
82	bro-c	79437	<	80507		356	89	98.3	90	84.4	105	28.9	2	50.1	77	63.7	117	25.1

83		80591	<	81019		142	90	99.3	91	95.8					78	72.5		
84	odv-e28	81052	<	81570		172	91	98.84	92	97.7	85	62.9	96	50.6	79	80.2	96	40.5
85	helicase	81527	>	85156	L	1209	92	99.83	93	96.5	84	46.8	95	41.8	80	78.7	97	27.8
86	odv-e25	85256	<	85906	L	216	93	100	94	94.9	82	64.5	94	45.1	81	83.9	98	48.0
87		85903	<	86388	L	161	94	100	95	98.8	81	69.7	93	51.0	82	88.4	99	32.9
88		86387	>	87145		252	95	99.6	96	94.8	80	57.5	92	51.4	83	82.5	100	37.9
89		87255	>	87770	E,L	171	96	97.7	97	91.2	77	33.9	142	34.7	84	31.7		
90	lef4	87802	<	89166		454	97	99.6	98	91.9	79	48.4	90	44.5	85	66.3	112	35.3
91	vp39	89165	>	90154	L	329	98	98.5	99	79.6	78	50.9	89	42.1	86	55.2	113	31.9
92	cg30	90237	>	91061	E	274	99	97.8	100	80.8	77	21.2	88	21.7	87	28.6		
93	91 k	91117	<	93555	L	812	100	99.3	101	92.9	76	44.4	83	41.5	88	58.4	121	28.3
94	tlp-20	93524	>	94111	L	195	101	98.0	102	91.9	75	41.4	82	31.4	89	63.7	122	41.2
95		93936	>	94658	L	240	102	99.2	103	82.8	74	62.3	81	49.5	90	68.3	123	51.5
96	gp41	94627	>	95628	L	333	103	100	104	97.9	73	55.7	80	57.1	91	80.4	124	36.0
97		95508	>	95963		151	104	100	105	92.2	72	41.5	78	35.9	92	58.3		
98	vlf-1	95965	>	97107	L	380	105	100	106	97.4	71	62.8	77	64.0	93	90.5	126	27.6
99	ctl	97104	<	97256	L	50	106	98	107	94.0			3	49.0			130	65.3
100		97329	<	98423	E	364	107	98.1	108	82.7	34	22.7			127	22.5		
101	p26	98524	<	99258	E	244	108	100	109	97.1	22	27.9	136	31.6	94	66.0		
102	iap-2	99307	<	100053		248	109	99.2	110	87.8	62	43.1	71	32.8	95	57.6	139	27.6
103		100010	<	100825		271	110	98.9	111	92.6	63	48.5	69	45.4	96	67.1		
104		100809	<	101174		121	111	100	112	95.9	64	55.1	68	47.9	97	75.6	137	40.4
105	lef3	101173	>	102354		393	112	99.5	113	82.2	65	30.5	67	27.0	98	54.3		
106	desmopl akin	102414	<	104675		753	113	99.2	114	79.9	66	24.9	66	22.3	99	29.9	135	38.2
107	DNA pol	104674	>	107676		1000	114	99.9	115	94.3	67	59.3	65	45.5	100	72.5	134	38.3
108		107710	<	108099	L	129	115	100	116	99.2	69	40.5	75	26.4	101	84.5		
109		108110	<	108367	L	85	116	100	117	100.0	70	70.6	76	41.9	102	87.1	128	32.9
110		108459	>	109199		246	117	97.2	118	86.0			151	61.5	103	39.0	10	32.9
111		109191	<	109736		181	118	98.9	119	87.9	57	31.8			49	23.6	81	29.5
112		109771	>	110232		153	119	98.7	120	96.1								
113		110287	>	110934	L	215	120	96.7	121	88.8					104	46.6	167	30.4
114	bro-d	110975	<	112033		352	121	89.6	122	83.6	105	23.4	2	45.1	123	39.3	117	27.9
115	bro-e	112087	<	112776		229	122	97.4	123	76.5	59	25.0	2	34.4	77	28.4	133	34.3
116	lef9	112859	<	114352	L	497	123	99.6	124	96.4	55	70.2	62	64.5	105	85.9	140	53.5
117	fp25	114430	>	115017	L	195	124	100	125	99.0	53	71.2	61	62.8	106	88.7	141	33.6
118	p94	115094	>	117598		834	125	99.4	126	82.5			134	41.7			20	35.8
119	bro-f	117622	>	118161	L	179	126	98.8	127	95.0	60	42.3			107	64.0	158	50.9
120	chaB2	118194	>	118469	L	91	127	100	128	96.7	52	55.2	60	48.8	108	67.4	103	38.6
121	chaB1	118447	>	118956		169	128	94.4	129	87.4	51	39.0	59	49.1	109	53.8		
122		118949	<	119428	E	159	129	98.7	130	95.0	50	40.3	57	37.4	110	59.4		
123		119678	<	119947	L	89	130	100	131	92.1	49	55.6	56	42.9	111	60.0		
124		119889	<	120098		69	131	100	132	95.8	48	49.3	55	42.0	112	71.6		
125	vp1054	120224	<	121234	E,L	336	132	100	133	93.2	47	53.2	54	40.3	113	67.3	173	35.0
126	lef10	121095	<	121322	L	75	133	98.7	134	93.3	46	47.2	53a	50.7	114	62.7		
127		121282	>	121509	L	75	134	100	135	93.3	45	36.0			115	50.8		
128		121523	>	122509	L	328	135	97.3	136	75.0	44	29.0			116	34.0		
129		122514	<	122987	L	157	136	100	137	94.3	43	57.4	53	51.2	117	66.9	169	28.8
130		122986	>	123489		167	137	100	138	88.6	42	26.5	52	20.8	118	49.1		
	hr3	123528		124601														
131	lap3	124860	>	125717	L	285	138	99.3	139	82.5	103	33.2	27	28.0	119	43.9	139	25.5

132	bjdp	125756	<	126910		384	139	98.7	140	88.0	39	31.5	51	19.5	120	36.6		
133	lef8	126931	>	129567		878	140	99.9	141	97.3	38	68.0	50	60.5	121	78.9	149	50.3
134		129595	<	130059		154	141	98.7	142	82.2					122	29.7		
135		130103	<	130303		66	142	100	143	95.4	37	25.4	43	32.3	124	58.6		
136	odv-e66b	130351	<	132342	L	663	143	98.6	144	88.8	96	33.8	46	27.9	125	50.2	150	35.4
137	p47	132390	>	133583		397	144	99.8	145	97.2	35	61.8	40	55.2	126	75.6	74	46.6
138		133594	<	134643		349	145	99.1	146	85.6					127	34.0		
	hr4	134722		135445														
139		135447	<	135710	E	87												
140		135947	>	136519	E	190	146	98.4	147	81.9					129	30.8		
141	bv-e31	136581	>	137285	E,L	234	147	100	148	94.9	33	66.5	38	63.3	130	85.0	77	42.4
142	lef11	137210	>	137584	L	124	148	99.2	149	90.2	32	49.0	37	39.1	131	64.6	51	38.1
143	39 k	137553	>	138407	L	284	149	99.3	150	92.0	31	30.3	36	33.8	132	49.8	50	30.2
144		138476	<	138673		65	150	98.5	151	86.2					134	37.1		
145	ubiquitin	138600	<	138902	L	100	151	100	152	93.9	28	77.6	35	77.9	135	94.7	47	76.6
146		138958	>	139503	L	181	152	98.9	153	93.4	27	50.3	34	35.5	136	63.8		
147		139854	<	140210	L	118	153	99.2	154	94.9	26	36.6	26	32.4	138	61.1		
148	dbp-2	140299	>	141279	E	326	154	99.1	155	95.4	25	42.8	25	24.2	139	59.9	87	24.0
149	lef6	141285	>	141710	L	141	155	98.6	156	93.7	24	45.7	28	25.5	140	47.1		
150		141751	<	141996		81	156	100	157	97.5			29	41.4	141	75.3		
151	p26	142112	>	142912	L	266	157	98.9	158	96.6	22	44.9	136	33.1	142	67.1		
152	p10	142951	>	143202	L	83	158	100	159	92.9	21	47.1	137	35.1	143	70.4	5	43.4
153	p74	143289	<	145262	L	657	159	99.4	160	94.7	20	55.3	138	52.5	144	65.9	72	39.3
154		145343	>	145594	E,L	83	160	98.8	161	91.8	19	32.1			145	53.0		
155	ie1	145631	<	147436		601	161	99.3	162	91.1	14	41.5	147	29.4	146	47.4		
156	ep23	147478	>	148053	L	191	162	100	163	95.3	13	31.4	146	33.5	147	63.9	9	30.9
157	chtB1	148114	<	148392	L	92	163	100	164	92.4	12	55.4	145	44.2	148	70.7	10	40.7
158	odv-e27	148395	<	149231	L	278	164	100	165	97.5	11	59.8	144	51.7	149	90.7	114	27.7
159	odv-e18	149270	<	149527	L	85	165	100	166	88.4	10	54.1	143	56.3	150	62.1	11	61.1
160	p49	149529	<	150914	L	461	166	100	167	97.4	9	57.4	142	49.2	151	78.0	12	35.9
161	ie0	150932	<	151636	L	234	167	99.6	168	95.7	8	42.7	141	30.8	152	64.5		
162	rr1	151802	<	154087	E	761	168	99.5	169	93.3					153	62.3		

Note. [a]Column 3 indicates ORF location and transcriptional direction on the HearMNPV genome. [b]Column 4 indicates the presence of early (E) and/or late (L) promoters located upstream of the start codon of each ORF. E indicates a TATA sequence followed by a CAGT or CATT mRNA start site sequence 20–40 nucleotides downstream, within 180 bp upstream of the start codon. L indicates the presence of a (A/T/G) TAAG up to 180 bp upstream of the initiation codon.

HearMNPV ORFs had an average length of 870 bp, with ORF85 (*helicase*) being the largest (3,627 bp) and ORF99 (*ctl*, conotoxin-like protein) being the smallest (150 bp). The 162 predicted ORFs encode 46,677 aa. The total coding sequence and intergenic regions were 139,026 and 15,170 bp, which represented 90.16% and 9.84% of the genome, respectively. The four *hrs* were distributed along the genome, with sizes ranging from 724 to 1,766bp, and their total sequence was 4,749bp, accounting for 3.08% of the genome. Thirty-eight ORFs overlapped with adjacent ORFs by between 1 and 244 bp, with a total of 1485 bp.

Of the 162 ORFs identified in HearMNPV, 21 possessed a consensus early promoter motif (a TATA box followed by a CAGT or CATT motif 20 to 40 bp downstream, and up to 180 bp upstream, of the initiation codon). Seventy-

one ORFs only contained a late promoter motif ((A/T/G) TAAG up to 180 bp upstream of the initiation codon), and nine had both early and late promoter motifs, which might allow transcription during both the early and late stages of infection. Sixty-one ORFs lacked any recognizable consensus early or late promoter motifs up to 180 bp upstream of the ATG. Eighty-six ORFs (46%) were oriented in a clockwise direction and 76 ORFs (54%) were in a counter clockwise direction, according to the transcription orientation of the polyhedron gene.

Comparison of HearMNPV ORFs to other baculoviruses

The overall gene arrangement and the homology between genes of the HearMNPV and other baculoviruses genomes were compared using Identity-GeneParity analysis [26]. The gene content and organization of HearMNPV were compared with a group I NPV (AcMNPV [32]), Group II NPVs (MacoNPV-B [33] MacoNPV-A [34], HearSNPVG4 [7] and AgseNPV [35]), and GV (HearGV [11]. HearMNPV shares 117 ORFs with AcMNPV, 161 ORFs with MacoNPV-B, 159 ORFs with MacoNPV-A, 123 with HearSNPV-G4, 147 with AgseNPV, and 89 with HearGV. The average amino acid sequence identities between HearMNPV and AcMNPV, MacoNPV-B, MacoNPV-A, HearSNPV-G4, AgseNPV and HearGV were 44.7%, 98.5%, 90.2%, 41.0%, 58.5%, and 38.6%, respectively.

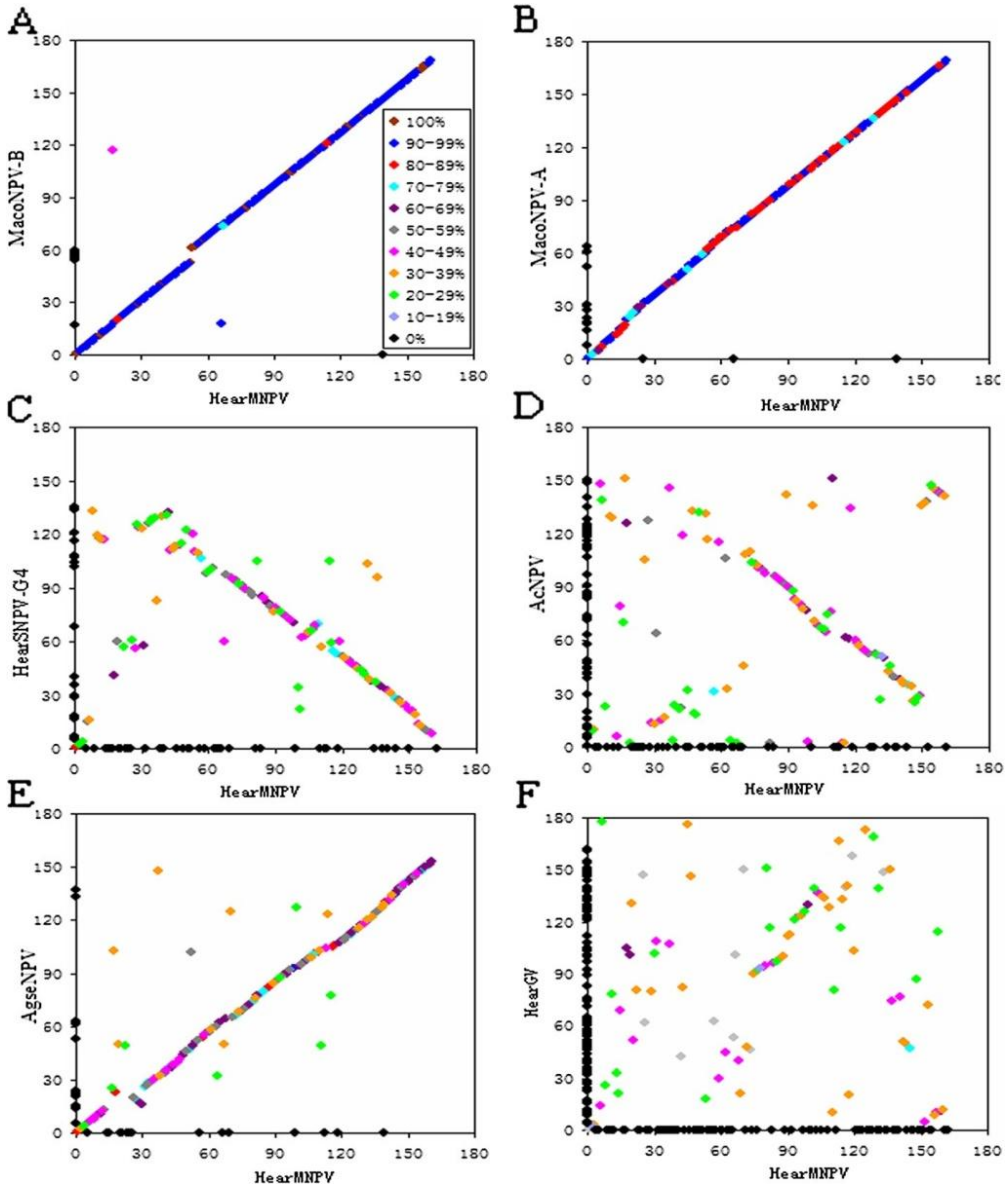
Comparison of the gene order between HearMNPV and MacoNPV-B revealed a significantly gap between *orf* 52 to *orf 53* in the HearMNPV genome. The gap corresponds to a region of MacoNPV-B comprising *orf54, 55, 56, 57, 58, 59*, and *60*. In addition, the *orf66* and *orf17* of HearMNPV are homologous to the *orf18* and *orf117* of MacoNPV-B with 99, 41.2% aa identity, respectively (Table 1). However, the locations of these homologues are not conserved. Relative to each other HearMNPV (x-axis) and MacoNPV-B (y-axis) contain 1 and 8 unique genes, respectively. However, HearMNPV and MacoNPVB maintain perfect co-linearity in gene content and arrangement (Pic. 4A).

The gene arrangement of HearMNPV was also completely collinear with that of MacoNPV-A. The result of the Identity-GeneParity analysis showed that relative to each other HearMNPV (x-axis) and MacoNPV-A (y-axis) contain 2 and 10 unique genes, respectively. There was also high collinearity between HearMNPV and MacoNPV-A (Pic. 4B).

In terms of gene content, arrangement, and homology level, HearMNPV is significantly distant from HearSNPVG4, although they infect the same host, H. armigera. Relative to each other HearMNPV (x-axis) and HearSNPV-G4 (y-axis) contain 38 and 18 unique genes, respectively, and these genes are distributed throughout the genomes (Pic. 4C). The ‘left’ part of the HearMNPV genome (ORF5-69) displayed a high degree of gene scrambling in the gene parity plot analysis. The homologous ORFs from HearMNPV 70 to 160 are approximately collinear with the HearSNPV-G4 ORFs 8 to 96; however, the direction of the diagonal indicates these regions are inverted, relative to each other, except for HearMNPV ORF102-107 (corresponding to HearSNPV-G4 ORF62–67) (Pic. 4 C).

Relative to each other HearMNPV(x-axis) and AgseNPV (y-axis) contain 15 and 11unique genes, respectively. The collinearity between HearMNPV and AgseNPV was higher than that between HearMNPV and HearSNPV-G4, and lower than that between HearMNPV and MacoNPV-B or MacoNPV-A. (Pic. 4 E)

The collinearity between HearMNPV and AcMNPV from Group I was lower than those between HearMNPV and NPVs from group II (Pic. 4D); the parity analysis of HearMNPV and HearGV ORFs displayed a much more dispersed pattern (Pic. 4 F).



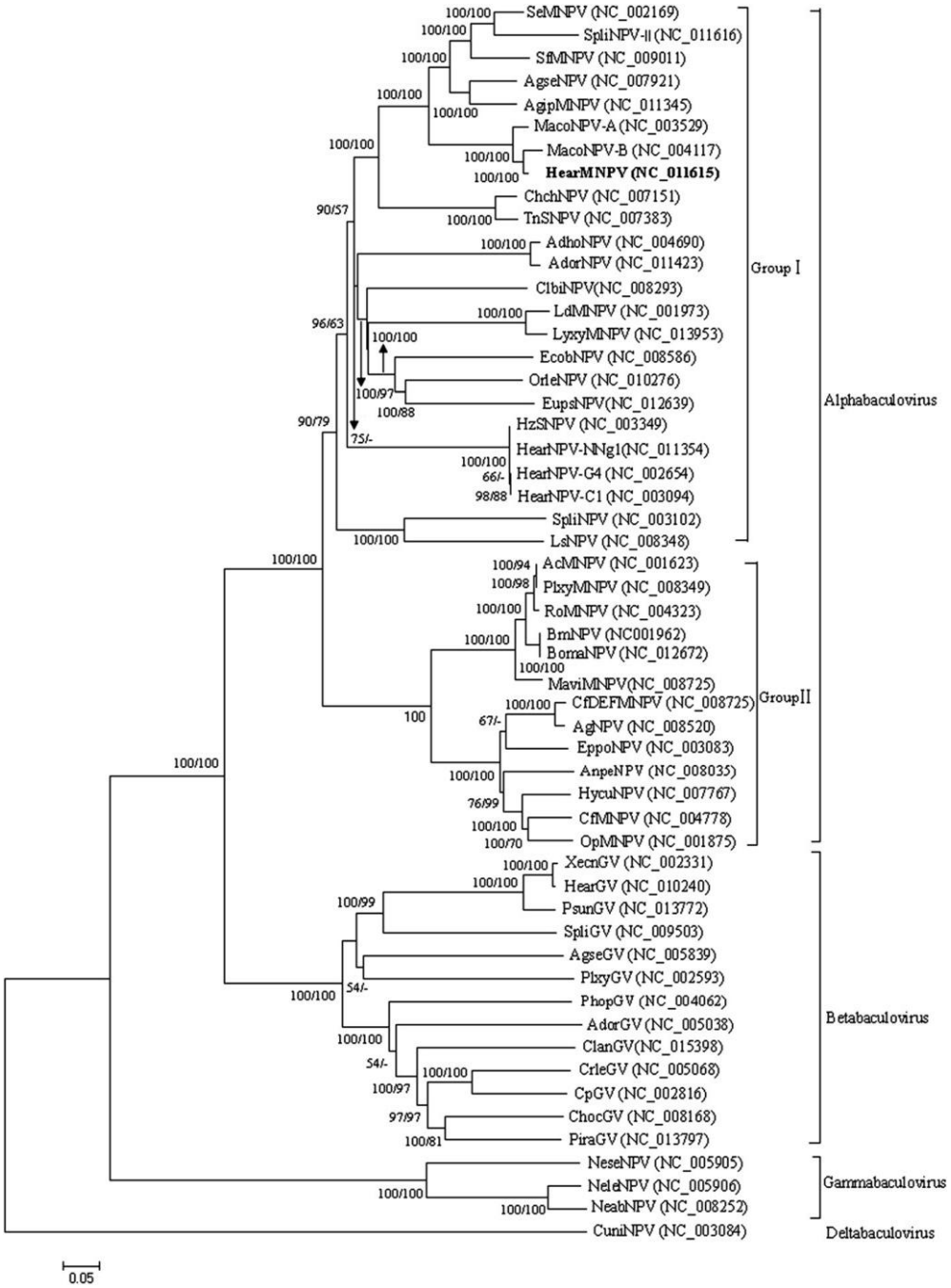
Pic. 4. Identity-Gene Parity Plotsof HearMNPV withMacoNPV-B(A),MacoNPV-A(B),HearSNPV-G4(C),AcMNPV(D), AgseNPV(E), andHearGV(F).Aminoacid identity (%) of individual homologousORFs of HearMNPV compared to other baculoviruses are shown in various colors. ORFs uniqueto each virus are placed on the x-axis and y-axis, respectively (black diamonds)

Phylogenetic analysis

Based on 29 concatenated, conserved genes [36], a phylogenetic tree was estimated for 54 baculoviruses. The results reflected the current systematic assignment of the viruses (Pic. 5), indicating that HearMNPV and MacoNPV-B are grouped together and are distinct from HearSNPV-G4 HearSNPV-C1 and HearSNPV-NNg1. In addition, the phylogenetic analysis of three highly conserved genes (*lef-8, lef-9*, and polh) indicated that the HearMNPV sequences were separated from the other eighteen HearMNPV

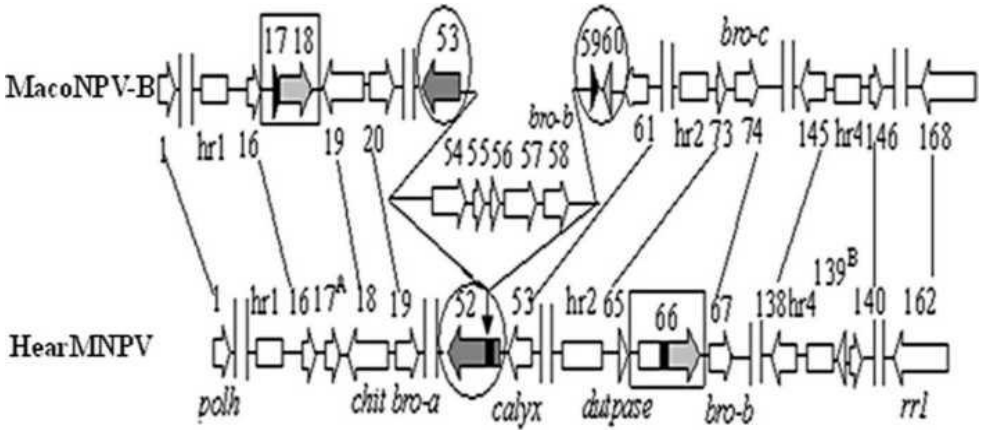
isolates [14]. These results imply that HearMNPV is a new isolate that differs from HearSNPV.

Genomic Comparison between HearMNPV and MacoNPV-B: HearMNPV lacks a 5.4-kb fragment that contains five ORFs



Pic. 5. Phylogenetic analysis of concatenatedamino acid sequencealignments,showing bootstrap values >50% for NJ and MPtrees at each node (NJ/MF).Thelocationof HearMNPV shownin bold.TheGenBankaccessionnumbersof eachvirusarelistedafterthenames.

Compared with the MacoNPV-B genome, the HearMNPV genome does not have a 5.4-kb fragment that contains ORF54, 55, 56, 57, and 58 (Pic. 6). The nucleotide identities between HearMNPV orf52 1–633 bp, 639–896 bp, and 853–1050 bp and MacoNPV-B *orf 53, orf 59, orf 60* are 98%, 98%, and 95%, respectively. Amino acids 147–349 of the protein encoded by HearMNPV *orf52* are 100% identical to those of the protein encodedMacoNPV-B *orf 53* and amino acids (aa) 1–65 of the protein encoded by HearMNPV *orf52* are 86% identical to the amino acid sequence of MacoNPV-B *orf 60* (Figure 6, indicated by the gray parts in the circles and arrows). However, there was no aa sequence identity between the proteins encoded by HearMNPV *orf52* and MacoNPV-B *orf 59* (Pic. 6, indicated by the black parts in the circles and arrows). The MacoNPV-A genome also lacked the 5.4-kb fragment, suggesting that an insertion in the genome might have lead to the division of ORF59 in MacoNPV-A[33].



Pic. 6. Comparison of the genome structure of HearMNPV and MacoNPV-B. The left and right arrows represent ORFs in HearMNPVand MacoNPV-Bgenomes, respectively. The numbers above the arrows represent the names of the ORFs in HearMNPVand MacoNPV-Bgenomes. The lines between the names of the ORFs represent homologies between the HearMNPVand MacoNPV-Bgenomes. The black region of arrows in the circle or box represent the nucleotide sequences homologies and the gray region of arrows in the circle or box represent the amino acid sequence homologies. The down arrow indicates the sites where the 5.4-k b fragment is inserted.The letter A indicates that the ORF of HearMNPVhas no homolog in the corresponding position of MacoNPV-B.The letter B representsthe ORF unique to HearMNPV.Double Vertical Lines represent ORFs that are not in the HearMNPVand MacoNPV-Bgenomes.

According to the sequence analysis of 54 whole genomes of baculoviruses, the 5.4-kb fragment present in MacoNPV-B but not in HearMNPV shared homologous sequences with XecnGV [30] and HearGV [11], by reverse alignment (Table 2). However, this phenomenon was not observed in other genomes. Combined with the phylogenetic analysis (Pic. 5), the results suggest that the 5.4-kb fragment was gained during evolution of MacoNPV-B genome, suggesting that these lineages were capable of infecting the same host species at some point during their history [33]. HearMNPV and HearGV could infect the same host cotton bollworm, *H. armigera*, which provides the opportunity for the natural recombination between two viruses. However, HearMNPV did not gain the 5.4-kb fragment from HearGV by recombination.

Table 2. **Comparison of ORFs aa identity from 5.4-kb fragment of MacoNPV-B, XecnGV and HearGV**

MacoNPVB	Homologoues (% aa id entity)				
	ORF54	ORF55	ORF56	ORF57	ORF58
XecnGV	ORF65(98)	ORF64(98)	ORF62(93)	ORF61(98)	ORF131(84) ORF60(60)
HearGV	ORF60(94)	ORF59(94)	ORF58(55)	ORF57(94)	ORF133(89) ORF54(50)

HearMNPV ORF66

The nucleotide sequence of ORF66 has high nucleotide sequence similarity to MacoNPV-B's ORF17 and ORF18. Presumably, a mutation gave rise to the division of HearMNPV ORF66 into two open read frames in MacoNPV-B.

HearMNPV ORF66, located between ORF65 (dutpase) and ORF67 (bro-b), is 1779 bp long and encodes a protein 592aa. The aa sequence identity is 99% between the first 301aa of HearMNPV ORF66′s (874–1779 bp) and MacoNPV-B ORF18 (301aa). However, the genome sequence of HearMNPV ORF66 and MacoNPV-B ORF18 are not collinear (Pic. 4A, Pic. 6).

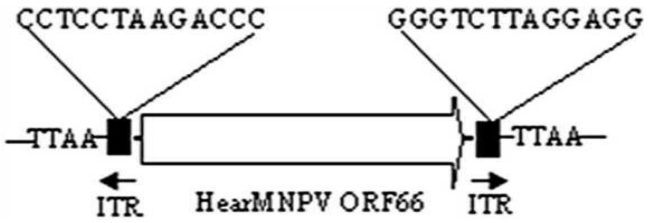
Nucleotides 676–968 of HearMNPV ORF66 are 95% identical to MacoNPV-B ORF17; however, the amino acids encoded by this nucleotide sequence did not share amino acid identity with the protein encoded by MacoNPV-B ORF17 because of frameshifts and other mutations of a few nucleotides. There is also no sequence similarity between the N-terminal region (1–675 bp) of HearMNPV ORF66 and MacoNPV-B, either at the nucleotide or amino acid level (shown in the boxes of Pic. 6).

The HearMNPV ORF66 protein is 92% identical to the five homologous *hr1, hr2, hr3, hr4*, and *hr5*, each of approximately 608aa in size, of Heliothis virescens ascovirus -3e (HvAV-3e) [37], and 85% identical to the proteins encoded by *orf34* (564aa) and *orf77* (606aa) of Spodoptera frugiperda ascovirus-1a (SfAV-1a) [38]. The comparison showed that these homologous ORFs have four conserved cysteine domains, suggestive of a zincbinding domain, hypothesized to be a DNA binding domain. This putative domain is found at the C terminus of a large number of transposase proteins, indicated that this might be related to gene duplication in the genome.

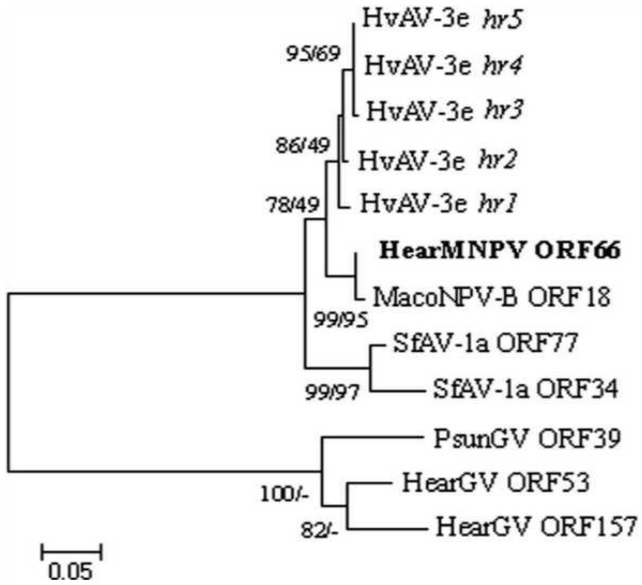
Interestingly, we have found an element in the right and left flanking DNA sequences of HearMNPV orf66 had two perfect inverted terminal repeats (ITRs) of 13 nucleotides. Moreover, the tetranucleotide5′-TTAA-3′, which is very common in transposition of the TTAA framily, is duplicated upon this element[39,40] (Pic. 7). This indicated that this element could insert exclusively at this insertion site (TTAA). However, the ORF66 has no amino acid identity with *piggyBac* transposase [41] by blastp analysis. Sequence analysis showed that the right and left flanking DNA sequences of HvAV-3e *hr1, hr2, hr3, hr4,* and *hr5* also have two perfect ITRs of 13 nucleotides. However, the left flanking DNA sequences of MacoNPVB *orf18* lacked the sequence CCTCCTAAGACCC. These results indicated homologous of HearMNPV *orf66* in MacoNPVB was split into MacoNPVB *orf18* and *orf17* during evolution.

Searching for homologs of HearMNPV ORF66 among the baculoviruses revealed that only HearMNPV ORF66 (592aa), MacoNPV-B ORF18 (301aa), HearGV ORF53 (572aa), ORF157 (572aa), ORF157 (576aa), and PsunGV ORF39 (571aa) are homologous ORFs. The phylogenetic analysis indicated that HearGV ORF53, ORF157, and PsunGV ORF39 belong to the same phylogenetic branch, while HearMNPV ORF66, MacoNPV-B ORF18, HvAV-3e hr1–hr5, and SfAV-1a ORF34 and 37 belong to the same phylogenetic clade (Pic. 8). HearMNPV and HvAV-3e are both isolated from cotton bollworms,

HearMNPV ORF66 and HvAV-3e hr1–hr5 share a flank structure, and have the highest amino acid identity among the homologous genes in baculovirus and ascoviridae to date (excluding unreleased relevant data). This data indicated that these genes might have been exchanged among species and genera.



Pic. 7. Diagram of the region with two DNA sequences flanking a putative Transposase ORF (HearMNPV °rf^{66}) of 1 779 bp encoding a protein with 592 amino acids. The tetranucleotideTTAA duplicated is characteristicof a transpositionevent by a transposable element. ITR representthe inverted terminalrepeats



Pic. 8. Phylogenetic analysis of the HearMNPV ORF66 amino acid sequence. The phylogenetic tree shows bootstrap values >50 % for NJ and MP trees at each node (NJ/MP). The location of HearMNPVORF66 is shown in bold. The sequences used are from Mamestraconfigurata NPV-96B (ORF18), Helicoverpaarmigera GV(HearGV(ORF53 and ORF157), Pseudaletiaunipuncta GV(ORF39),Heliothisvirescensascovirus 3e (HvAV-3e hr1^{-} hr5), and Spodopterafrugiperdaascovirus 1a (SfAV-1a ORF34 and ORF77).

The genomic differences between HearMNPV and MacoNPV-B are mainly located between hr1 and hr2, including the deletion of the 5.4 kb fragment in HearMNPV and the changes in ORF66, both of which were close to a bro gene (Pic. 6).

HearMNPV ORF17

The locations of the HearMNPV ORF17 and its homologue in the MacoNPV-B genome are not conserved. HearMNPV ORF17 only has 41.2% aa identity to MacoNPV-B ORF117(e = 6e^{-46}, with 98% query coverage), while HearMNPV ORF110 was collinear at an aa identity of 97.2% with MacoNPV-B ORF117, indicating that HearMNPV ORF17 has no significant collinearity with the homologous ORF of MacoNPV-B.

HearMNPV unique ORF

HearMNPV ORF139 is 264 bp long and encodes a protein of 87 aa. There is an early promoter CATT motif in the 180 bp region upstream of the start codon. Using both BLASTX and BLASTP searching, no homologous protein was found among baculoviruses.

Bro genes

The occurrence of the baculovirus repeat ORF (bro) gene family is a striking feature in many baculovirus genomes[42]. bro genes are associated with regions of viral genome rearrangement [43]. BmNPV BRO proteins have nucleic acid binding activity that influences host DNA replication and transcription [44]. BRO proteins function as nucleocytoplasmic shuttling proteins that utilize the CRM1mediated nuclear export pathway [45]. We identified six bro genes dispersed among the genome of HearMNPV and named them bro-a to bro-f, according to the order of their appearance on the linearized genome. There are eight and seven bro genes in MacoNPV-A and MacoNPVB, respectively. The bro genes are classified into four groups, based on the similarity of the 41-amimo acid core domain sequences used for LdMNPV BRO protein classification [46]. HearMNPV bro-c, bro-d, and bro-e belong to group I bro genes, bro-a and bro-b belong to group II bro genes, bro-f belongs to group IV There is no bro gene corresponding to MacoNPV-B bro-b, which belongs to group III. The HearMNPV genome also lacks homologs of the MacoNPV-A bro-a (group I) and bro-c (groupIII) genes.

The HearMNPV *bro-a, -b, -c, -d, -e, - f* genes showed aa identities of 83%, 77.5%, 98.3%, 89.6%, 97.4%, and 98.8% to MacoNPV-B *bro-a, -c, -d, -e, -f, -g*, respectively. MacoNPV-B bro-b is located in the region of 5.4 kb fragment of MacoNPV-B, which is lack in the HearMNPV genome.

The HearMNPV *bro-a* gene had an N-terminal region from aa 1 to aa 134 with aa identities of 63% and 95% to MacoNPV-B *bro-a* and MacoNPV-A *bro-b*, respectively. The C-terminal region, from aa 135 to aa 331, has aa identities of 98% and 93% to MacoNPv-B *bro-a* and MacoNPV-A *bro-b*, respectively. This suggested that *bro-a* C-terminal regions are the highly conserved portions in these three virus genomes.

HearMNPV *bro-f* shows high homology to a hypothetical protein P20 [47] from *Leucania separata* NPV (LeseNPV) and MacoNPV-A *bro-h*, which both encode 179 aa proteins with amino acid identities of 95% and 98%, respectively. HearMNPV *bro-f* shows the highest homology to MacoNPV-B *bro-g*, with an amino acid identity of 98.8%. However, amino acids 1–17 of HearMNPV *bro-f* are not found in MacoNPV-B *bro-g*.

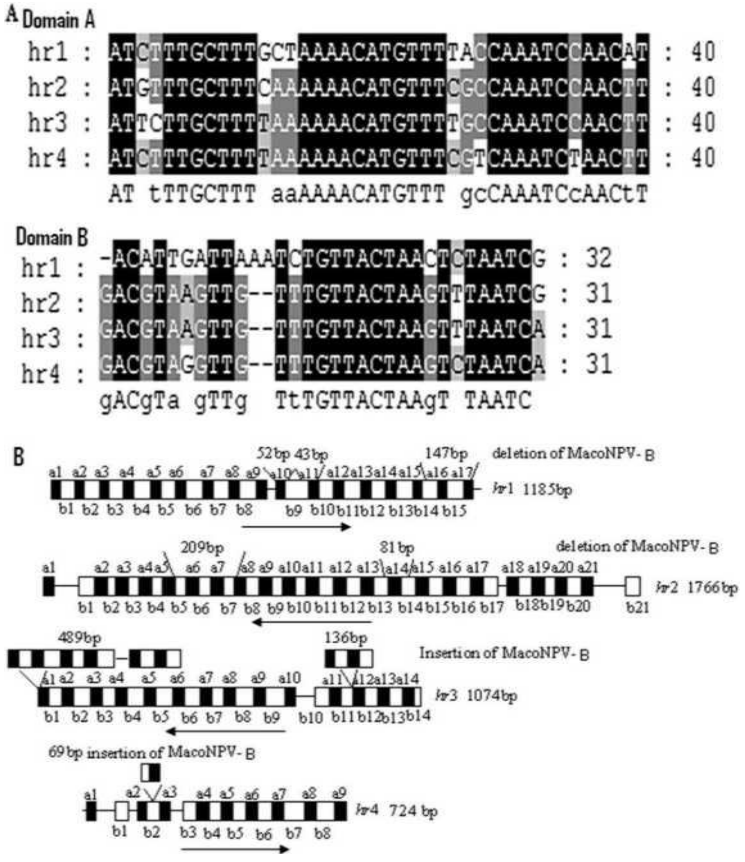
When comparing the *bro* genes of HearMNPV with MacoNPV-B, the lowest aa identity is between HearMNPV *bro-b* and MacoNPV-B *bro-c*, at 77.5%. The HearMNPV ORF66 gene is adjacent to HearMNPV *bro-b* and has changed much comparing with ORF17 and ORF18, which are closest to MacoNPV-B *bro-c*.

The differences between HearMNPV *bro-c, d*, and *e* and their homologs in MacoNPV-B represent minor nucleotide insertions, deletions, and substitutions.

The *bro* genes of HearMNPV differed from those of MacoNPV-B in both sequence and number, which indicated that the bro gene region is one of the most important in genomic variation of baculoviruses. The differences between HearMNPV and MacoNPV-B (the 5.4 kb fragment and the location of ORF66) were found in the vicinity of a *bro* gene. These differences indicated that *bro* gene might play a role in gene exchange, and, consequently, viral virulence and host range.

Hrs

Variable numbers of hr sequences, composed of direct repeats containing a "core" imperfect palindrome and dispersed unevenly among the genome in AT rich intergenic regions, have been identified in most baculovirus genomes [48]. The baculovirus hrs act as enhancers of RNA polymerase II-mediated transcription of baculovirus early promoters [49], as well as functioning as origins of DNA replication in transient replication assays [50,51]. They are also sites of frequent recombinant and rearrangement in baculovirus genomes [52,53]. Four hrs were identified in the HearMNPV genome, with the sizes of 1185 bp (*hr1*), 1766 bp (*hr2*), 1074 bp (*hr3*) and 724 bp (*hr4*), respectively. The *hrs* are distributed throughout the HearMNPV genome: between *orf14* and *orf15*, *orf63* and *orf64*, *orf130* and *orf131*, and *orf138* and *orf139* for *hr* 1, 2, 3, and 4, respectively. Sequence analysis confirmed that the four hrs comprise two apparent domains with perfect or near-perfect 40 bp palindromes (designated type A) and 31 bp flanking repeats (designated type B) at the head/end of one or both sides of the palindromes (Pic. 9A). Each hr repeat sequence comprises two apparent domains (type A and type B) that is similar to that described for MacoNPV-A and MacoNPV-B [33,34]. However, the repeat unit numbers in each hr of HearMNPV was different from MacoNPVA and MacoNPV-B.



Pic. 9. Comparison the[hr] regionsbetweeHearMNPVndMacoNPV-B'aneA: a deducedonsensuequencef domainA andB fromeachh regionwasusedFort hisalignmenConserveiequencesr indicatew ithdiffereretiading: l ackndicatel0(% conservatio n, gray>70% conservatioandno shading 70% conservaticPianeB: arfowsepresertti edirectioandpositionsftherepeat-Andrepeat-B regions, l ack^oxes'epreserty peA repeatandblanlb oxes'epreserty peB repeats

The four hrs of HearMNPV are located at similar positions in the genome as those of MacoNPV-B and MacoNPV-A. Sequence alignment between HearMNPV and MacoNPV-B hrs indicated that these four homologous regions had some insertions/deletions of different sizes, giving rise to identities of 92.0%, 92.3%, 86.6%, 81.9%, respectively. *hr1* has three insertions, two of 52 bp and 43 bp that contained only a type A repeat, and one of 147 bp that contained type A and type B repeats. *hr2* has two insertions (209 bp and 81 bp) that contained both type A and type B repeats. *hr3* has the biggest deletion (489 bp) and another large deletion of 136 bp, which also contained both type A and type B repeats. *hr4* has a small deletion of 69 bp that also contains both type A and type B repeats (Pic. 9B). The HearMNPV *hr4* (724 bp) is shorter than the MacoNPVB *hr4* (1178 bp) occurrence, probably caused by the presence of HearMNPV ORF139, which is adjacent to HearMNPV hr4. HearNPV NNg1 contains five *hrs* (hr1-hr5), similar to HearNPV C1, G4, and HzNPV. The arrangement of these *hrs* on the genome is almost the same in HearNPV C1, G4 and HzNPV, and it is possible that variability in the hr sequences affect not only progeny virus production, but also the insecticidal activity of the *Helicoverpa* spp. NPVs [9]. The homologous regions are also suggested to be responsible for the difference in virulence between two *Mamestra configurata* NPV-A variants, v90/4 and v90/2 [54], indicating that the difference in the organization of the homologous regions of HearMNPV and MacoNPV-B are possibly associated with mechanisms of recombination.

Conclusion

HearMNPV differs significantly from HearSNPV not only in biological properties and morphology, but also in gene content, arrangement, and homology level based on genome sequence comparison, which considered to be different viruses, and not variants of the same virus. Although the average amino acid sequence identity between HearMNPV and MacoNPV-B is 98.5%, but their effective host range are different. Moreover, a 5.4-kb segment of the MacoNPV-B genome which is the apparent result of recombination with an ancestor of XecnGV is absent in the HearMNPV genome, suggesting that the recombination event responsible for the occurrence of this 5.4 kb segment occurred after the divergence of MacoNPV-B and HearMNPV. The location and length of HearMNPV *orf66* and MacoNPV-B *orf18* are different in their respective genomes. Phylogenetic analysis indicated that these events may occur after MacoNPV-B and MacoNPV-A separated from their ancestor. These distinct differences between HearMNPV and MacoNPV-B may account for their different host range.

Acknowledgements

This work was supported by grants from the National Natural Sciences Foundation of China (31070139) and the “973” Project (2012CB114602).

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EXPERIMENTAL USE OF *CHOUIOIA CUNEA* EULOPHIDE TO PROTECT FORESTS

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白蛾周氏啮小蜂实验在森林保护中的应用

摘要：白蛾周氏啮小蜂是一种能够快速寻找寄主并高效防治鳞翅目害虫的蛹寄生蜂。

俄罗斯已经开始了这种寄生蜂的饲养和森林病虫害防治应用技术研究。本文章重点讨论了白蛾周氏啮小蜂第一次应用于模毒蛾（*Lymantria monacha* Linneaus）害虫的防治试验。模毒蛾1758年曾在莫斯科地区爆发成灾。研究结果表明，白蛾周氏啮小蜂可以找到模毒蛾并成功杀死其蛹。下一阶段应用技术研究将确定该寄生蜂的最佳释放速率。

关键词：白蛾周氏啮小蜂，模毒蛾，森林保护。

ЭКСПЕРИМЕНТАЛЬНОЕ ПРИМЕНЕНИЕ ЭУЛОФИДА *CHOUIOIA CUNEA* ДЛЯ ЗАЩИТЫ ЛЕСА

Эулофид *Chouioia cunea* Yang, 1989 является высоко эффективным куколочным паразитоидом, способным находить и успешно заражать куколок многих видов вредителей леса из отряда Чешуекрылых, или Lepidoptera.

В России начаты работы по разработке технологии разведения и применения этого паразитоида для защиты леса от ряда вредителей. В статье описан первый опыт применения этого паразитоида в очаге массового размножения монашенки *Lymantria monacha* Linneaus, 1758 в Московской области. Первый опыт выпуска показал, что эулофид способен находить и успешно уничтожать куколок монашенки. Следующим этапам в разработке технологии его применения станут исследования по установлению оптимальных норм выпуска паразитоида.

Ключевые слова: эулофид *Chouioia cunea,* шелкопряд-монашенка, защита леса.

ABSTRACT: The possibility of infection parasitoid pupae *Chouioia cunea Lymantria monacha* in the centers of mass breeding in Moscow and Vladimir regions. It is shown that in natural conditions parasitoid can find and infect the pupae of this pest. Development of this parasitoid rearing and application technology to protect forests against a number of pests have commenced in Russia. This paper highlights this parasitoid 1st application experience in nun moth *Lymantria monacha* Linneaus, 1758 mass outbreak in Moscow region. Next step in its application technology development will be studies of this parasitoid optimal release rates.

Key words: eulofid Chouioia cunea, nun moth, the protection of forests.

One of the key areas in biological forest protection is application of so called biological procedure opportunity that is parasite or predatory entomophage application. This area has a long background however in our country its application was limited by low scale experiments.

One of forest protection agents can be a pupae parasitoin *Chouioia cunea* Yang, 1989 (Hymenoptera: Eulophidae) that is an efficient entomophage of a number of scale-winged insects.

Many countries developed eulophide mass rearing and application technologies for plant protection based on laboratory rearing on various host insects [1-4]. Thus in China Chinese oak silkworm (pic.1) is used more often. In Ukraine – big bee moth, in Iran – American white moth.

Pic. 1. Chinese oak silkworm papa infected by eulophide (bright ellow mass – small eulophide larvae in pupa cavity).

In development of domestic small scale production technology we used big bee moth *Galleria mellonella* Linnaeus, 1758 (Lepidoptera: Pyralidae) and *Samia cynthia ricini* Boisduval, 1854 (Lepidoptera, Saturniidae) pupae. It enabled production of eulophide species sufficient quantity and start its testing in natural conditions.

It is essential that lab-reared entomophage after release in mass outbreaks can successfully find and infect pupae. Experimental entomoparasitoid release was done in 2013-2014 to check man-made eulophide species ability in nun moth *Lymantria monacha* Linneus, 1758 (Lepidoptera, Erebidae) mass outbreaks in forests of Moscow (hic. 2) and Vladimirskaya regions (table 1).

Pic. 2. Larch damage in nun moth outbreak in the Paretskoe forest sub-district, Moscow region.

Table 1. **Entomophage release locations.**

Release year and location	Forest characteristics	Outbreak brief characteristics	Released parasitoid quantaty
2013, Moscow region, Paretskoe forest sub-district, rcompartment 83, stratum 8	Plantations of 1871, composition 10Лц+С, spruce in undergrowth.	70-100% damage of key canopy, and 80-100% - undergrowth	32.0 thousand pcs.
2014 Vladimirskaya region, Gorokhovetskoe forest sub-district compartment 56.	10С, aged around 100 years, pine in undergrowth	30-60% damage of key canopy, 40-70% -undergrowth	30.0 thousand pcs.

Before eulophide release in nun moth outbreak in the Paretskoe forest sub-district the pupae were collected to assess pest infection rate by local entomophages and efficiency of nun moth pupae lab infection. Eulophide imago were planted on them and its condition was surveyed in 2 weeks (table 2).

Table 2. **results of nun moth pupae collected in nature infection with *Ch. cunea***

Pupae number, pcs	Emerged moths, %	Disease affected, %	Зараженность паразитоидами, %		
			Diptera order	Hymenoptera order	*Chouioia cunea* (successful evolution/ dead pupae*)
135	11,9	1,5	8,9	5,9	4,4 / 67,4

* – dead nun moth pupae after *Ch. cunea* infection.

Thus in lab conditions the eulophide is able to kill most nun moth pupae without any impact on its infection level by local entomophages (pic.3).

Pic. 3. Lab *Ch. cunea* infected nun moth pupa

Lab eulophide infection of nun moth pupae results in eulophide complete evolution and its imago emergence only in 4,4% of cases, most part of *Ch. cunea* infected pupae (67,4%) died but the eulophide couldn't evolve in it completely. Nun moth eulophide infection had no impact on local parasite insect evolution in it with total mortality 14,8%. There was nun moth emergence since phytophage collection moment its pupae were at various morthogeny stages and *Ch. cunea* infection ahdn't affected moth evolution in pupae where moths had already shaped. Disease-related mortality (fungus entomophatogenes) was 1,5%.

In 2013 experiment nun moth pupae were collected in release locations and 100 m from it for lab analysis of infection rate by entomoparasitoid introduced in forest community. Collection was random on lower undergrowth spruce branches, totally 774 phytophage pupae were picked (table 3).

Table 3. **Results of nun moth prepupae (PP) and pupae (P) lab analysis after entomophage release in 2013.**

Sampling Nu.	Number in sampling, pcs .			Emerged mothsи, %	Disease affectedи, %	PP and P mortality due to local entomophaged,%	Pupae mortality due to *Chouioia cunea, %*
	всего	предкуколок (ПК)	куколок (К)				
1	120	30	31	48,3	0	51,7	0
2	111	37	18	50,5	0	49,5	0
3	140	40	33	47,9	0,7	47,1	4,3
4	60	29	9	36,7	1,7	61,6	0
5	126	41	17	54,0	0,8	43,7	1,5
6	87	18	11	66,7	0	31,0	2,3
7	130	16	27	66,9	0	28,5	4,6

The findings showed that in natural environment eulophide is able to find and infect nun moth pupae (pic. 3) that proves a principal opportunity of its application to kill nun moth pupae. Infected pupae die while in natural conditions there was no successful evolution of *Ch. cunea* and imago emergence. Dea eulophide larvae were found in dead nun moth pupae.

In 2013 and 2014 nun moth pupae were collected in release location 14 days after release. Collection was done at random on spruce undergrowth totally 573 pupae were picked (table 4).

Table 4. **Results of nun moth pupae lab analysis after entomophage release in the Vl;adimirskaya region**

Number in sampling, pcs	Emerged mothsи, %	Disease affectedи, %	mortality due to local entomophaged,%	Pupae mortality due to *Chouioia cunea, %*
573	48,3	0	51,7	3,67

Parasitic 2-winged prevailed in entomophage package, local parasitoid species identification was not done. Infected pupae die while eulophide successful evolution til imago stage was not found.

Results in 2014 proved 2013 ones and showed principal opportunity of entomophage application to kill nun moth pupae. Infected pupae die while eulophide successful evolution til imago stage was not found.

Thus eulophide release will not result in its accumulation in forest and its population growth. Entomophage release in nun moth outbreaks may be applied as a proactive preventive treatment. Thus *Ch. cunea* application is feasible at low phytophage population level when outbreak development is forecast in 1-2 years. In these conditions single or double application can prevent outbreak development and remove forest damage risk.

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ASSESSMENT OF RED PINE SAWFLY NUCLEAR POLYHEDROSIS VIRUS STRAIN EFFICIENCY

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松黄叶蜂核型多角体病毒毒株的效率评估

摘要：松黄叶蜂（*Nedoprion sertifer* Geoffroy）是广泛分布和危害严重的松树害虫之一，特别是对俄罗斯种植的小松林具有大的危害。最早的病毒制剂virin-diprion
常被用来防治松黄叶蜂幼虫，然而，这种病毒已经有10多年没有生产应用了。因此，需要开发一种新的基于昆虫肠道多角体病毒的防治这种害虫的制剂。初步的高毒力菌株筛选试验结果表明其防治效果显著，这种新的病毒生物制剂是从保存在国家病毒资源中心的菌株中筛选出来的，目前已经进行了小批量生产。

关键词：松黄叶蜂，核型多角体病毒，森林保护

ОЦЕНКА ЭФФЕКТИВНОСТИ ШТАММОВ ВИРУСА ЯДЕРНОГО ПОЛИЭДРОЗА РЫЖЕГО СОСНОВОГО ПИЛИЛЬЩИКА

Рыжий сосновый пилильщик *Nedoprion sertifer* Geoffroy является одним из наиболее широко распространенных и опасных вредителей сосны, особенно искусственных сосновых молодняков, в России. Ранее для защиты от его личинок часто использовали вирусный препарат вирин-диприон, который, однако, уже более 10 лет не производится. Поэтому возникла необходимость разработки нового современного средства защиты леса от этого вредителя на основе вируса полиэдроза кишечного типа. Описаны результаты проведенного поиска эффективных штаммов, их оценки и первичных испытаний. В результате проведенных работ выбран штамм, который задепонирован в Государственной коллекции вирусов и на его сонове начато малотоннажное производство нового вирусного биологического средства.

Ключевые слова: рыжий сосновй пилильщик, вирус полиэдроза, защита леса.

ABSTRACT: Red pine sawfly *Nedoprion sertifer* Geoffroy is one of the most wide spread and hazardous pine tests particularly in young pine plantations in Russia. Earlier virus preparation virin-diprion was often applied in protection against its larvae however it is out of production already over 10 years. Thus there was a need to develop an updated forest protection agent against this pest based on intestinal polyhedrosis virus. Efficient strain search findings, its primary trial evaluations are highlighted. It resulted in selection of the strain that was deposited at the Government virus collection and it served as a basis for new virus biological agent small tonnage production

Key words: red pine sawfly, polyhedrosis virus, forest protection.

Introduction

Red pine sawfly (RPS) *Nedoprion sertifer* Geoffroy is one of the most wide spread and hazardous pine tests in particular young pine plantations in Russia (Gurjanova et al, 2009). Earlier virus preparation virin-diprion was often applied in protection against its larvae however it is out of production already over 10 years (Golosova, Gninenko, 2009).

Searh of efficient nuclear polyhedrosis virus isolates in this pest mass outbreaks in some regions of our country has been done for development of a new preparation against this sawfly. Strain selection was based on previous collections of the pathological material stored at the VNIILM collection and further on virus isolates extracted from live populations.

Totally 9 virus isolates were picked for testing (table 1).

Primarily 5 virus isolates were tested (Sergeevga et all., 2010). It was found that it is reasonable to use 3 strains – NsrO98, NsrR11, NsrUS85 in the follow-up work. Next step of studies is aimed at these starins maintenance and activity testing in the following passages as well as bio-testing of new preparations produced from native populations in other regions of the country.

Table 1. **Virus isolate origin for bio-testing**

№№	**Pathological material origin**	**Strain working name**
1	Dry RPS dead bodies killed by virus infection from the phytophage mass outbreak in the Sholokhovskoe forest district, Rostovaskay region (collection of 2011)	S_{Nsr}R11
2	Dry RPS dead bodies killed by virus infection from the phytophage mass outbreak in the Ust-Donetskoe and Serafimovich forest districts< Volgogradskay region (collection of 2011)	$S_{Nsr}V_l$11
3	Virus isolate suspension extracted from 3 collections of RPS dead bodies (collection of 2009 in the Volgogradskay region)	$S_{Nsr}V_l$09
4	Virus isolate suspension extracted from 3 collections of RPS dead bodies (E.Orlovskaya collection stored since 1998)	S_{Nsr}O98
5	Neochek-S (powder) – US produced preparation developed against RPS (stored since 1985 in Orlovskay collection)	S_{Nsr}US85
6	RPS dead bodies killed by virus infection in lab conditions, picked in the phytophage mass outbreak in the Antipovskoe forest district, Rostovskay region	S_{Nsr}R12
7	RPS dead bodies killed by virus infection in lab conditions, picked in the phytophage mass outbreak in the Izobilnenskoe forest district, Stavropolsky territory	S_{Nsr}St12
8	RPS dead bodies killed by virus infection in lab conditions, picked in the phytophage mass outbreak in the Pravdinsky forest district, Moscow region	S_{Nsr}Mos12
9	RPS dead bodies killed by virus infection in lab conditions, picked in the phytophage mass outbreak in the Ilovlevskoe forest district, Volgogradskay region	S_{Nsr}Vl12

Materials and procedures

RPS larvae were collected in the phytophage outbreaks in the Volgogradskay and Moscow regions for lab work. The larvae brought to lab were nursed.

Test insect larvae infestation for assessment of selected isolate and strain activity was done twice. 1st infestation of the Volgogradskay population larvae was done with the strains S_{Nsr}O98, S_{Nsr}US85 and the isolates S_{Nsr}St12 и S_{Nsr}R12. 2nd infestation was conducted on the Moscow population larvae with the strains S_{Nsr}O98, S_{Nsr}R11/12, S_{Nsr}US85, S_{Nsr}St12P and the isolates S_{Nsr}Mos12, S_{Nsr}Vl12. Before infestation working suspensions were prepared: polyhedrosis concentrations were counted under microscope, centrifugation cycle was done if needed to produce bio-testing results applicable for comparison. As a result suspension titers were aligned according to plohedrosis content with regard to available biomass volume for each strain.

Small forest site experiments were conducted on growing pine trees. Branches with feeding larvae were treated. Treatments were done at 6 a.m. and 18 p.m. in dry windless weather.

Results and discussion

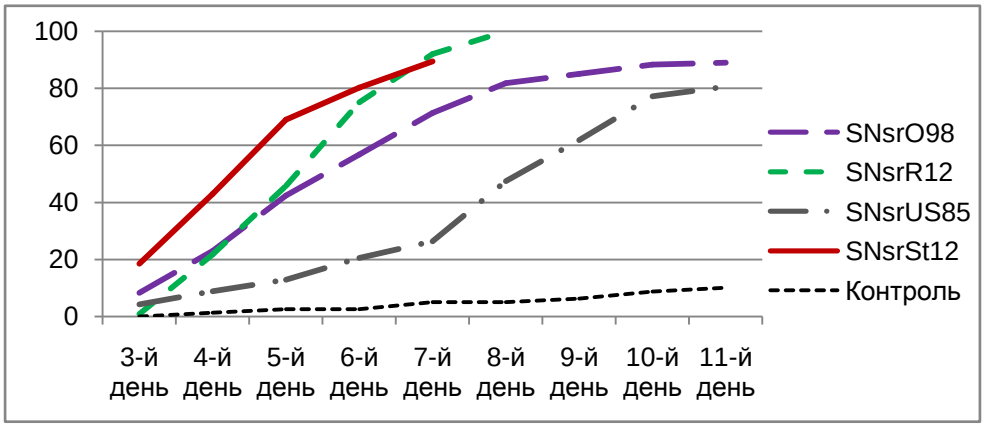
Findings of virus testing on the Volggogradskaya population larvae showed (table 2) the most efficient in larvae mortality rate was the isolate S_{Nsr}St12, after its treatment mortality completed on the 7th day but efficiency in maximum concentration was 89,4%, fast larvae feeding termination (4th day) and its early cocoon development were observed; with virus suspension twofold dilution cocoon number doubled while with fourfold dilution feeding intensity was higher and mortality was 95,7%.

Table 2. **Results of selected strain testing on the Volgogradskaya population larvae**

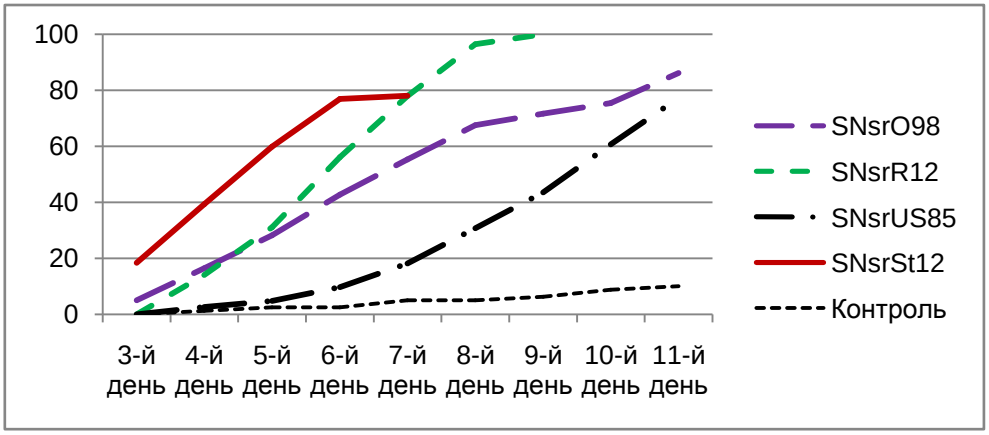
Strain/isolate	Concentration	Efficciency on the 5th day after treatment,%	Mortality period (day) start/end	Share of cocooned species, %
S_{Nsr}O98	$0,5x10^9$	42,36	3/11	9,9
	$0,24 x10^9$	28,21	3/11	12,5
	$0,13 x10^9$	4,33	3/11	26,9
S_{Nsr}R12	$0,3 x10^9$	45,70	3/8	0
	$0,15 x10^9$	31,05	4/9	0
	$0,06 x10^9$	4,79	3/9	3,3
S_{Nsr}US85P	$0,28 x10^9$	12,82	3/11	17,5
	$0,13 x10^9$	4,79	4/11	20,5
	$0,06 x10^9$	0,94	4/11	11,1
S_{Nsr}St12	$0,4 x10^9$	68,97	3/7	10,1
	$0,19 x10^9$	59,83	3/7	20,8
	$0,09 x10^9$	68,64	3/7	4,1

After treatment with the strains S_{Nsr}O98 и S_{Nsr}US85 larvae mortality was the longest – 11 days. Efficiency fourfold dilution of the strain S_{Nsr}O98 suspension was only 67,3%, cocoon development of almost 1\3 larvae was observed, 2,5% of species continued its evolution, at maximum concentration mortality was 89%, at twofold dilution – 86,1%, al survived species evolved in cocoon stage.

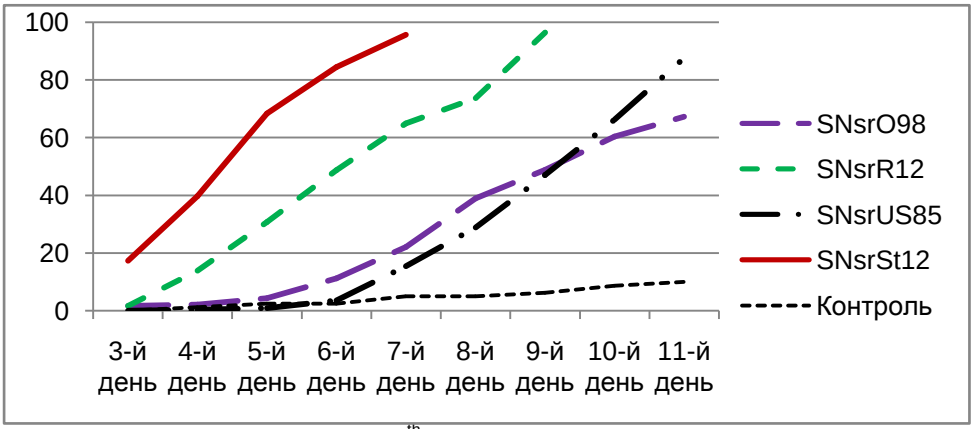
The Volgogradskay population larvae mortality dynamics after treatment with the tested viruses with different concentrations varied (pic. 1,2 and 3).



Pic. 1. Volgogradskaya population 4th age RSP larvae mortality after spraying with virus strains at maximum concentrations.



Pic. 2. Volgogradskaya population 4th age RSP larvae mortality after spraying with virus strains with suspension twofold dilution.



Pic. 3. Volgogradskaya population 4th age RSP larvae mortality after spraying with virus strains with suspension fourfold dilution.

The most efficient in this lot was the isolate S_{Nsr}R12: early cocoon development was observed at minimum concentration (4,6% of species); at

twofold dilution and maximum concentration infestation resulted in 100% species mortality on 9th and 8th days respectively. In control early cocoon development was observed just in 1 case. Mortality rate of larvae treated with S_{Nsr}O98 и S_{Nsr}R12 varied notably depending on titer nut final species mortality was registered on the same day both for minimum and maximum concentrations proving that the induced infection process development doesn't depend on concentration. Applied concentrations of the isolate S_{Nsr}US85 equally affect infection process evolution. Concentration levels of the S_{Nsr}St12 working solution didn't affect species mortality intensity. Larvae mortality was rather prolonged in time since work was done on larvae of various ages.

This testing stage resulted in association of S_{Nsr}R11 and S_{Nsr}R12 in one strain named S_{Nsr}R11/12.

Next passaging was tested on the Moscow population larvae with the strains S_{Nsr}O98P, S_{Nsr}R11/12P, S_{Nsr}US85P, S_{Nsr}St12P and the isolates S_{Nsr}Mos12, S_{Nsr}Vl12 (table 3). Larvae mortality was less prolonged in time compared to virus testing data on the Volgogradskaya population larvae that is linked to larvae younger age. Larvae mortality due to treatment with strains alredy after one passage was more intensive.

Table 3. **Selected strains 2nd passage results on the Moscow population larvae.**

Strain	Concentration	Efficiency on the 5th day after treatment, %	Mortality period (days) start\end	Share of cocooned species, %
S_{Nsr}O98	0,27x10^9	51,87	3/6	0
	0,13 x10^9	48,94	3/8	0
	0,05 x10^9	29,11	3/8	0
S_{Nsr}R11/12	0,35 x10^9	82,42	3/6	0
	0,17 x10^9	61,18	3/6	0
	0,08 x10^9	41,77	3/7	0
S_{Nsr}US85	0,29 x10^9	64,85	3/6	0
	0,15 x10^9	42,62	3/8	0
	0,08 x10^9	8,95	3/9	0
S_{Nsr}St12	0,26 x10^9	75,33	3/6	0,8
	0,12 x10^9	43,46	3/7	2,5
	0,056 x10^9	24,89	3/8	0
S_{Nsr}Mos12	0,34x10^9	66,24	3/7	0
	0,17 x10^9	54,43	3/8	1,7
	0,07 x10^9	40,93	3/8	0
S_{Nsr}Vl12	0,26 x10^9	84,81	3/7	0
	0,12 x10^9	23,21	3/8	2,5
	0,06 x10^9	14,77	3/9	1,7

As in the previous experiment the most efficient in larvae mortality rate was the strain S_{Nsr}R11/12, after minimum concentration treatment mortality ended on the 7th day, efficiency of all concentrations was 100%, larvae feeding termination was observed on the 9th day and all larvae died without cocoon development.

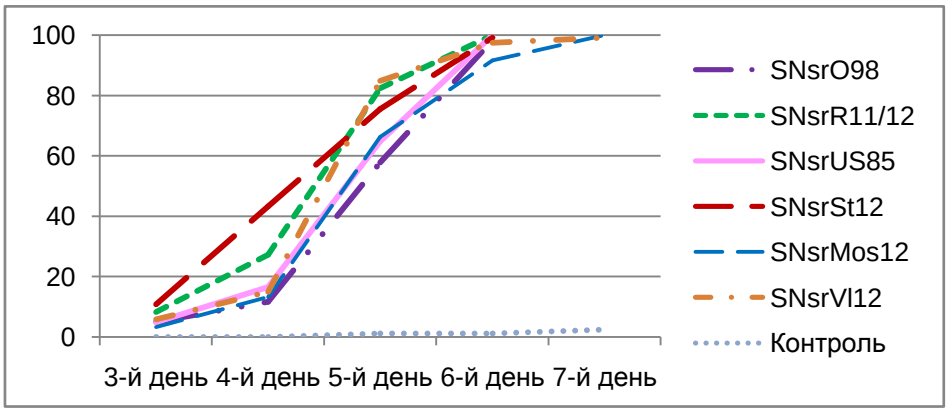
The longest larvae mortality was after treatment with the strains S_{Nsr}US85 and S_{Nsr}Vl12 at fourfold dilution larvae main share mortality ended on the 9th day. At minimum concentration of the strain S_{Nsr}Vl12 with fourfold

dilution cocooning of 1.7% of species was observed, with twofold dilution of this strain suspension 2.5% of species passed into cocoon stage.

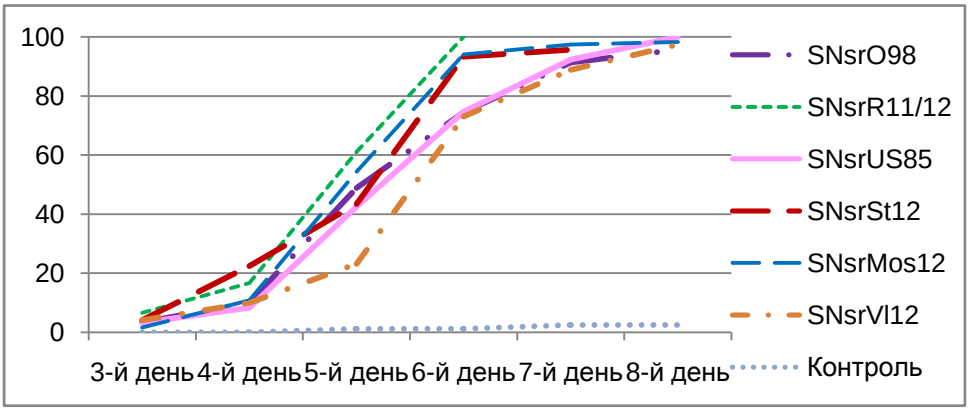
As well cocooning was observed after treatments with the isolates S_{Nsr}Mos12 and S_{Nsr}St12 in options with dissolved concentrations.

After treatment with the suspension S_{Nsr}O98 at all concentrations there was survival of sawfly larvae that continued feeding. Survived larvae were observed in options with minimum concentrations of the strain S_{Nsr}US85 and S_{Nsr}St12 suspensions. In control early larvae cocooning was observed just in one case.

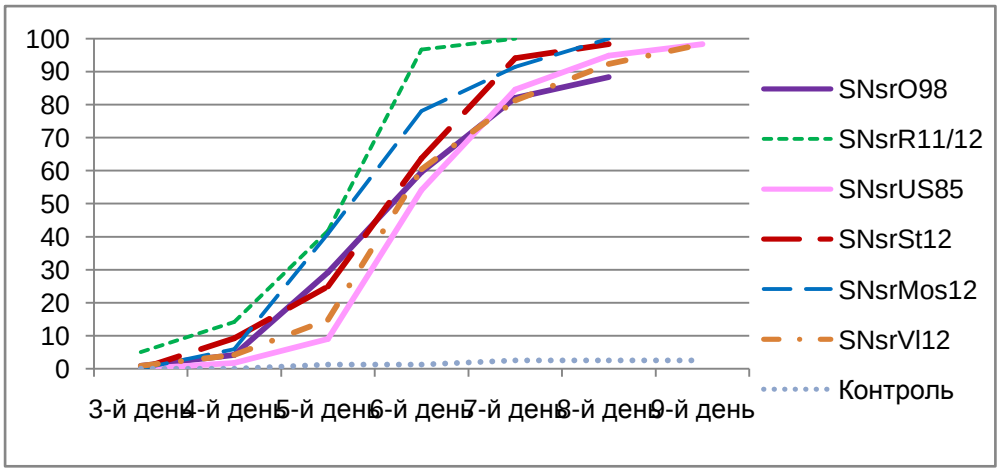
The Moscow population larvae mortality dynamics after treatments with the tested strains at various concentrations showed available general regularity (pic. 4, 5 and 6) – in all options mortality increased with growth of virus particle concentration in working suspension. Variations in larvae response to gradually falling suspension concentrations were sharper with fourfold dilution. In all options at maximum concentration mortality was more intensive that with twofold and fourfold dilution that indicates high virulence of the tested strains. Prolonged larvae mortality with rather high final efficiency in options with lower concentrations shows infection process evolution in test insect species. With minimum virus particle content in suspensions larvae mortality intensity decreased. In control larvae mortality didn't exceed 2,5% over the whole observation period that proves high survival of the Moscow population,



Pic. 4. The Moscow population RPS 3-4 age larvae mortality after spraying with the virus strains at maximum concentration.



Pic. 5. The Moscow population RPS 3-4 age larvae mortality after spraying with the virus strains with twofold dilution.



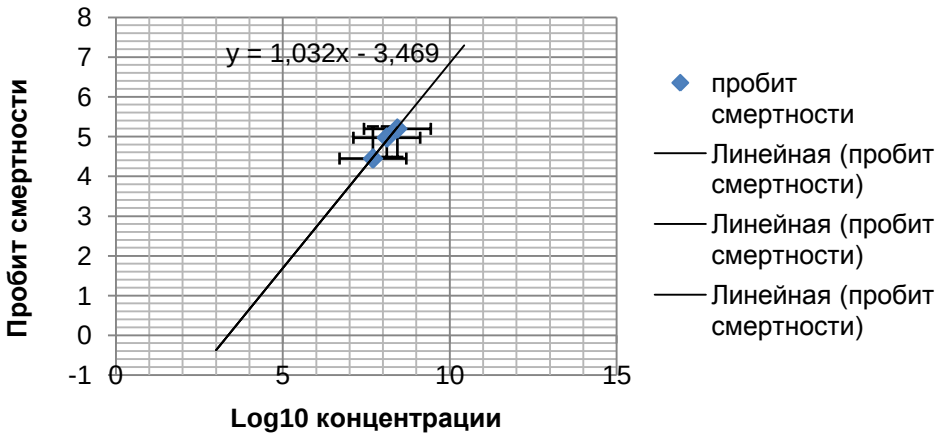
Pic. 6. The Moscow population RPS 3-4 age larvae mortality after spraying with the virus strains with fourfold dilution.

Comparison of the selected virus strain application efficiency was done in relation to average weighted efficiency calculated for each working suspension concentration. For that sum of each strain final efficiencies was divided by tested strain number. Deviation from average efficiency was derived as a difference between larvae mortality in option and average efficiency in concentration. Thus «-» sign shows that strain efficiency is lower than average weighted one and «+» sign respectively higher (table 4).

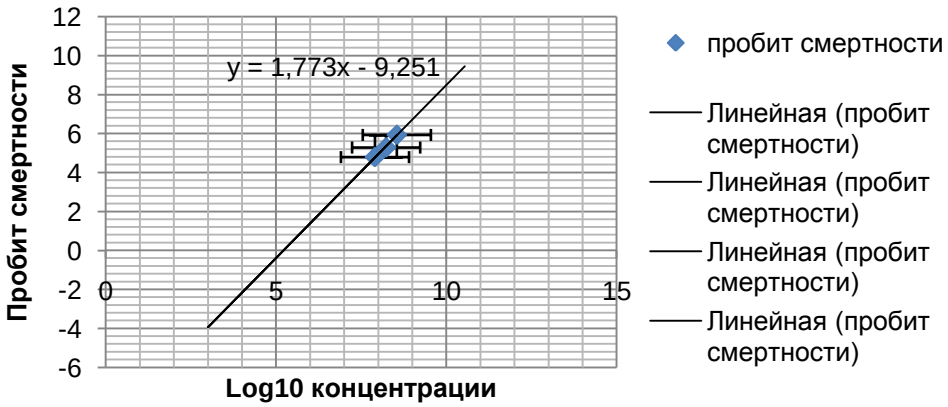
Table 4. **Deviation of strain isolate efficiency from average one.**

Strain isolate	Concentration	Deviation from average efficiency of nuclear polyhedrosis virus
S_{Nsr}O98	fourfold dilution	- 9,12
	twofold dilution	- 2,17
	maximum	- 1,13
S_{Nsr}R11/12	fourfold dilution	+ 2,85
	twofold dilution	+ 2,14
	maximum	+ 0,56
S_{Nsr}US85	fourfold dilution	+ 1,13
	twofold dilution	+ 2,14
	maximum	+ 0,56
S_{Nsr}St12	fourfold dilution	+ 1,14
	twofold dilution	- 2,13
	maximum	- 0,28
S_{Nsr}Mos12	fourfold dilution	+ 2,85
	twofold dilution	+ 0,43
	maximum	- 7,88
S_{Nsr}Vl12	fourfold dilution	+ 1,14
	twofold dilution	- 0,42
	maximum	- 0,29

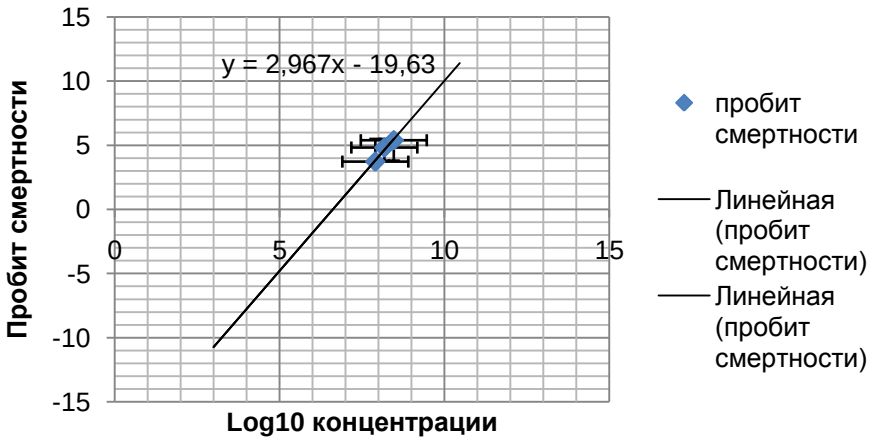
The findings processing based on probit analysis has been done for final conclusions on selection of the most efficient strains. For “dose-impact” dependence graphing transformed data was produced. It resulted in regression equations for “dose-impact” dependence for each strain and standard errors (pic. 7 - 12).



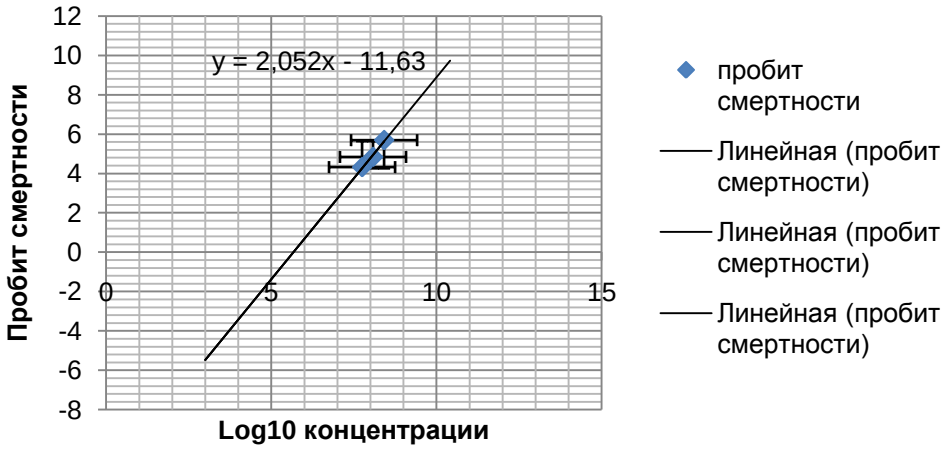
Pic. 7. “Dose-impact” dependence after treatment of the Moscow population RSP larvae with the strain S_{Nsr}O98P.



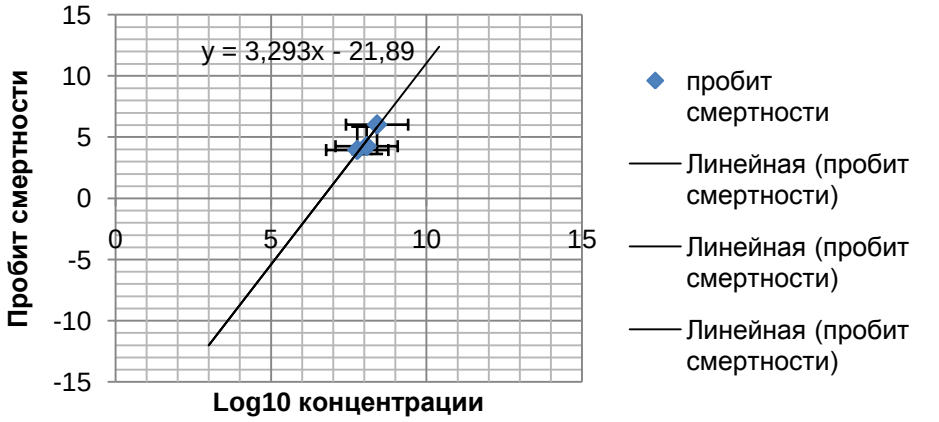
Pic. 8. “Dose-impact” dependence after treatment of the Moscow population RSP larvae with the strain S_{Nsr}R11/121.



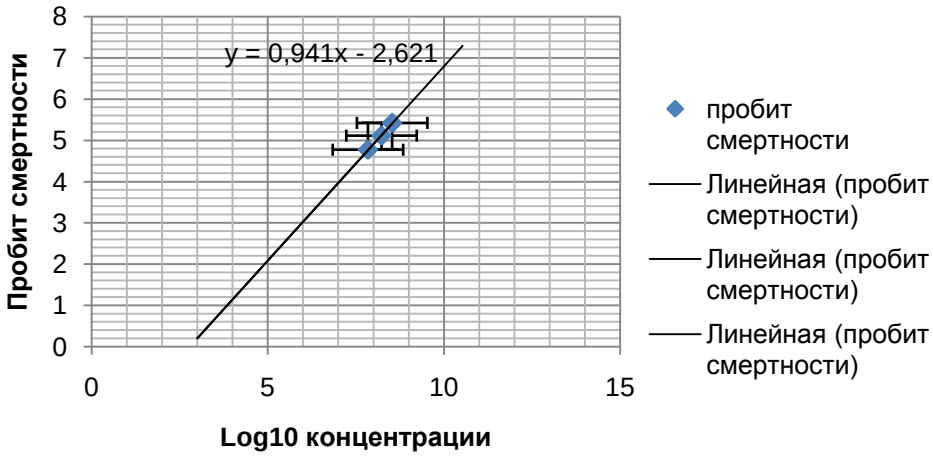
Pic. 9. “Dose-impact” dependence after treatment of the Moscow population RSP larvae with the strain S_{Nsr}US85.



Pic. 10. “Dose-impact” dependence after treatment of the Moscow population RSP larvae with the strain S_{Nsr}St12



Pic. 11. “Dose-impact” dependence after treatment of the Moscow population RSP larvae with the isolate S_{Nsr}Mos12.



Pic. 12. “Dose-impact” dependence after treatment of the Moscow population RSP larvae with the isolate S_{Nsr}Vl12.

Based on the derived equations expected mortality probits and expected mortality percentage values have been calculated for each strain. Actual χ^2 values have been calculated for each strain and on this basis the strains with inaccurate bio-testing were rejected.

Calculations of LC_{50} and LC_{95} values for the tested isolates and strains were based on the derived regressive equations.

It was found that LC_{50} value for all tested strains was similar. Meanwhile calculation showed that for the strains S_{Nsr}O98P and S_{Nsr}Mos12 isolates LC_{95} value sufficiently exceeds this indicator in other options. Therefore S_{Nsr}O98P and S_{Nsr}Mos12 virulence is way below.

As a result in final selection of the tested isolates and strains for follow-up field trials they were compared taking into consideration test result parameters, isolate efficiency with regard to each concentration, larvae mortality period in an option (infection process evolution period in option with minimum concentration was accepted), early cocooning after larvae infestation, value of lethal concentration that induce 95% mortality, χ^2. Criteria accuracy. The tested isolate was rejected if two or more parameters gave unacceptable result (table 5).

Table 5. **Comparison of the selected isolates and strains.**

Strain isolate	**Deviation from mean efficiency**	**Mortality period**	**Cocooning**	**LC_{95}**	**Bio-testing accuracy**	**Field trials feasibility**
S_{Nsr}O98	Below average	8	no	$LC_{95}= 6,4x10^9$	yes	no
S_{Nsr}R11/12	Above average	7	no	$LC_{95}= 0,9x10^9$	yes	yes
S_{Nsr}US85	Above average	9	no	$LC_{95}=0,7x10^9$	yes	yes
S_{Nsr}St12	Below average	8	yes	$LC_{95}= 0,8x10^9$	yes	no
S_{Nsr}Mos12	Below average	8	yes	$LC_{95}= 7,1x10^9$	yes	no
S_{Nsr}Vl12	Above average	9	yes	$LC_{95}= 0,4x10^9$	no	no

This data shows that the strain S_{Nsr}O98 shouldn't undergo field trials since its efficiency is below average weighted one and LC_{95} is high. It is unreasonable to use the strain S_{Nsr}St12 since its efficiency is below average weighted one and part of infected species transformed into a cocoon stage. However since other parameters of the strain S_{Nsr}St12 gave good results the decision to test its efficiency once again in biomass production process was made. The isolate S_{Nsr}Mos12 was rejected since its efficiency is below average there was early cocooning and LC_{95} is high. The isolate S_{Nsr}Vl12 was rejected because prolonged larvae mortality period, observed early cocooning and bio-testing is inaccurate. The strain S_{Nsr}US85 gave only prolonged larvae mortality so it was selected for field operations. The strain S_{Nsr}R11/12P should be used as well since all parameters in this option are acceptable.

Cultivation of the most virulent strains in old age RPS larvae has been done to buildup virus material volume. Simultaneously the selected strain efficiency was assessed.

The strain S_{Nsr}St12 showed low efficiency. Mortality dynamics of larvae infected with it is extended in time and a great number of species transformed into cocoon stage was observed. The strain was rejected.

Polyhedra were counted in dead bodies of 3-4 age larvae treated with all tested strains irrespective of its follow-up rejection (table 6).

Table 6. **Virus mass output (larvae equivalent "LE") after infestation of the Moscow population 3-4 age larvae.**

№ №	Strain	Mortality period, days	Larva average mass, g.	Larvae equivalent	LC_{95}
1	S_{Nsr}O98	8	0,0430	$0,09x10^9$	$LC_{95}= 6,4x10^9$
2	S_{Nsr}R11/12	7	0,0352	$0,09x10^9$	$LC_{95}= 0,9x10^9$
3	S_{Nsr}US85	9	0,0445	$0,11x10^9$	$LC_{95}=0,7x10^9$
4	S_{Nsr}St12	8	0,0649	$0,15x10^9$	$LC_{95}= 0,8x10^9$
5	S_{Nsr}Mos12	8	0,0573	$0,13x10^9$	$LC_{95}= 7,1x10^9$
6	S_{Nsr}Vl12	9	0,0538	$0,1x10^9$	$LC_{95}= 0,4x10^9$

The findings showed that polyhedra output after treatments with all tested strains is comparable in quantity and directly depends on larvae size. Therefore this indicator essential in general in selection of strains for industrial applications isn't defining in our case. The findings prove validity of lab selection of the strains S_{Nsr}R11/12 and S_{Nsr}US85 – they have the lowest LC_{95} values and sufficient LE.

Testing of the strains S_{Nsr}US85 and S_{Nsr}R11/12 showed its high efficiency even on late age larvae. After treatment with S_{Nsr}R11/12 in most cases mortality started on the 3th day after treatment while larvae stopped feeding a day before dying. As a rule mortality ended on the 7^{th} day after treatment. Insect mortality in infestations with the strain S_{Nsr}US85 started only on the 4^{th} day main part of dead bodies was collected on the 7^{th} day.

Thus high biological activity of the strains S_{Nsr}US85 and S_{Nsr}R11/12 has been proved and virus biomass for field trials prepared.

Small site field experiments were conducted in the Rostovskaya, Orenburgskaya and Moscow regions.

The findings show that growth of dead larvae share practically in all experiment treatments depended on applied virus suspension concentration. So application of suspensions with spore content $1x10^9$, $0,5x10^9$, $0,25x10^9$ triggered larvae mortality on the 4^{th} day after treatment respectively:

In S_{Nsr}R11/12 option

– the Orenburgskaya region: 66,7%; 62,87%; 57,19%;
– the Rostovskaya region: 19,05%; 15,71%; 14,65%;
– the Moscow region: 53,65%; 43,67%; 40,64%.

In S_{Nsr}US85 option

– the Orenburgskaya region: 60,6%; 48,97%; 44,58%;
– the Rostovskaya region: 8,66%; 9,61%; 1,71%;
– the Moscow region: 95,08%; 84,19%; 72,28%.

Larvae mortality rate depended on treated larvae age in experiment.

In the Orenburgskaya region 2-3 age larvae mortality individually started on the 3^{rd} day. Complete mortality came on the 5^{th} day. After treatment with the strain S_{Nsr}R11/12 complete termination of feeding was noted on the 4^{th} day and in S_{Nsr}US85 option 30% of species continued feeding. After application of S_{Nsr}R11/12 suspension at minimum tested concentration mortality on the 4^{th} day was 57,19%; for S_{Nsr}US85 – 44,58%.

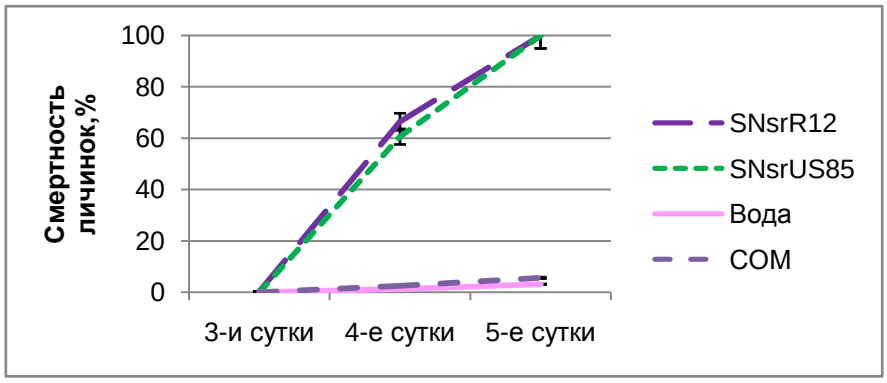
In the Rostovskaya region treatment of 4 age larvae resulted in 100% mortality on the 6^{th} day. Here larvae stopped feeding just partially on the 4^{th} day, its main part dropped needle consumption on the 5^{th} day. On the 4^{th} day mortality was for S_{Nsr}R11/12 – 14,65%; and S_{Nsr}US85 – 1,71%.

In the Moscow region treatment was done when larvae were in 1-2 age. Here its mass mortality started on the 3^{rd} day and after treatment with the strain

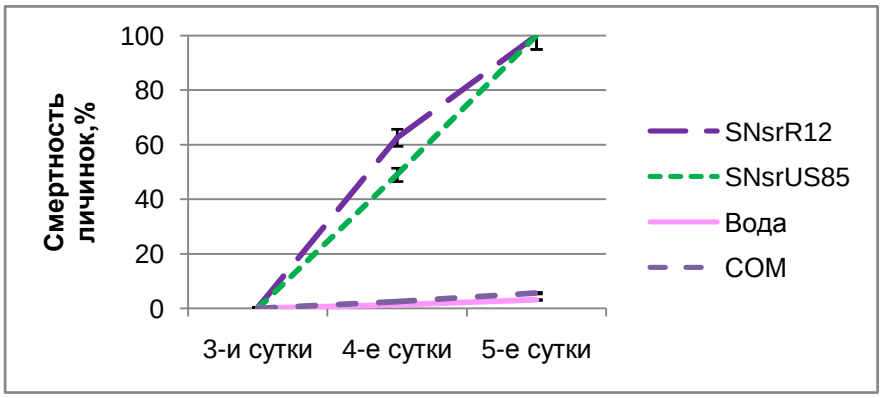
S_{Nsr}R11/12 ended on the 4th day after spraying and in S_{Nsr}US85 option total mortality came on the 5th day. Note that there were 1 age larvae individually on treated branches in all tested strain concentrations. Total mortality of such species came on the 3rd day independent of experiment option.

Early cocooning wasn't observed in any experiment.

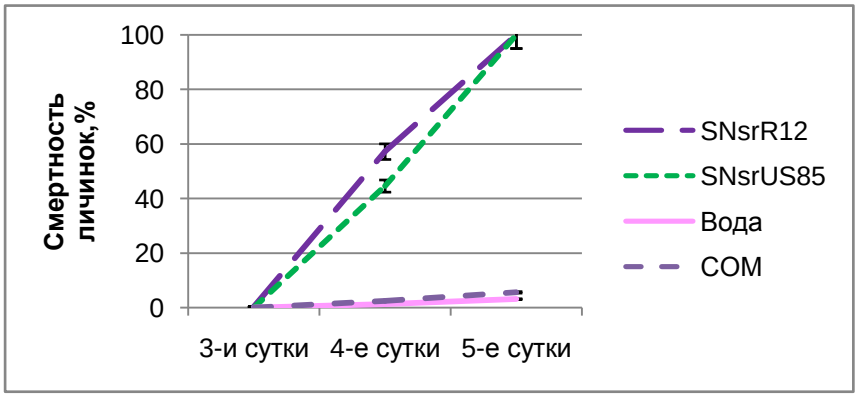
Different age larvae mortality dynamics in tested concentrations varied sufficiently (pic. 13-22).



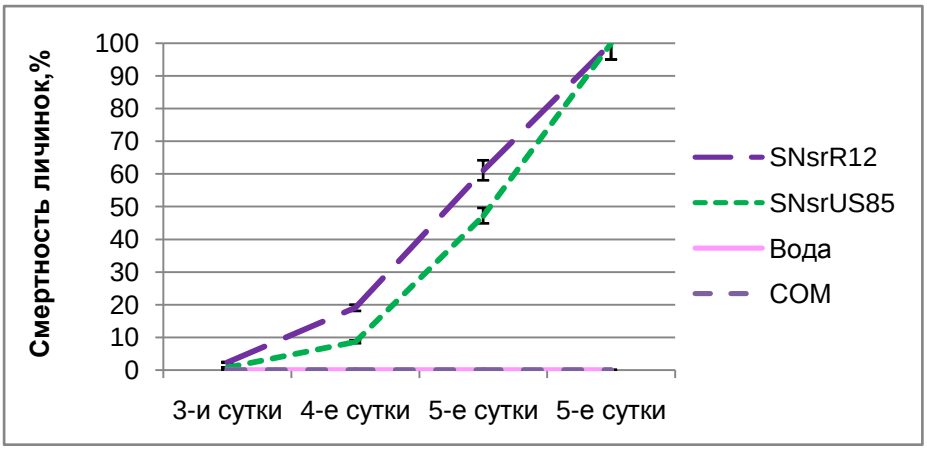
Pic. 13. RPS 2-3 age larvae mortality in the Orenburgaskaya region after virus strain spraying in concentration $1x10^9$



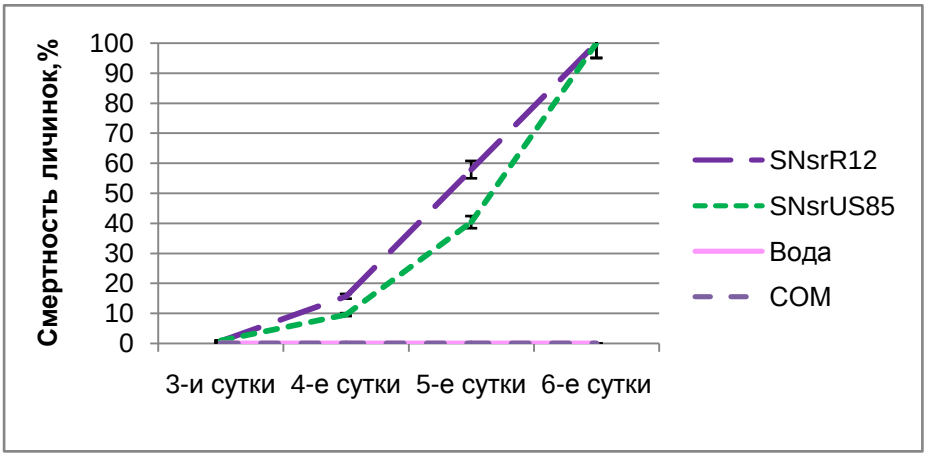
Pic. 14. RPS 2-3 age larvae mortality in the Orenburgaskaya region after virus strain spraying in concentration $0,5x10^9$



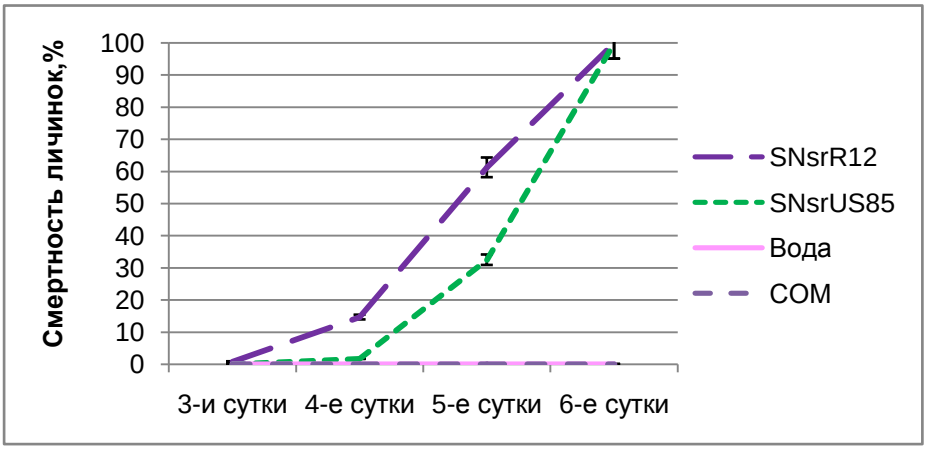
Pic. 15. RPS 2-3 age larvae mortality in the Orenburgaskaya region after virus strain spraying in concentration $0,25x10^9$



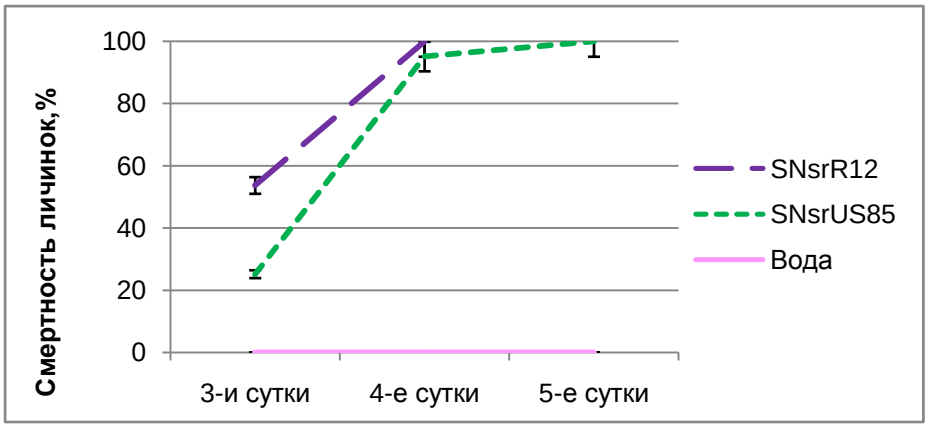
Pic. 16. RPS 4 age larvae mortality in the Orenburgaskaya region after virus strain spraying in concentration $1x10^9$



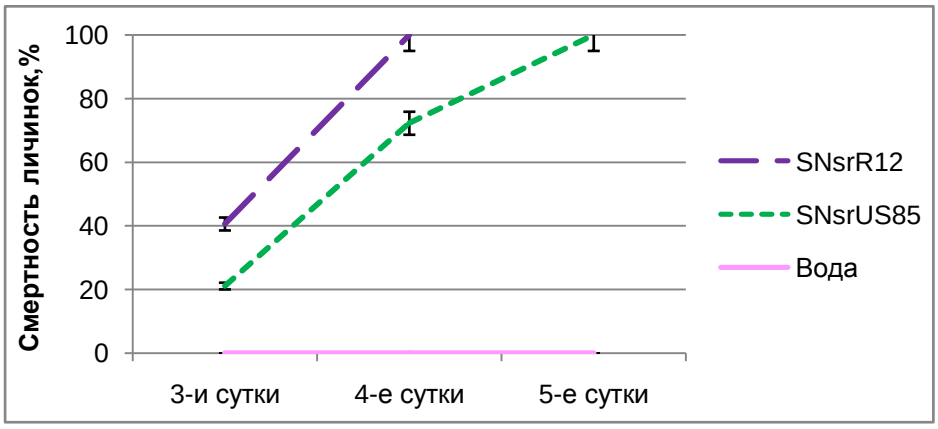
Pic. 17. RPS 4 age larvae mortality in the Orenburgaskaya region after virus strain spraying in concentration $0,5x10^9$



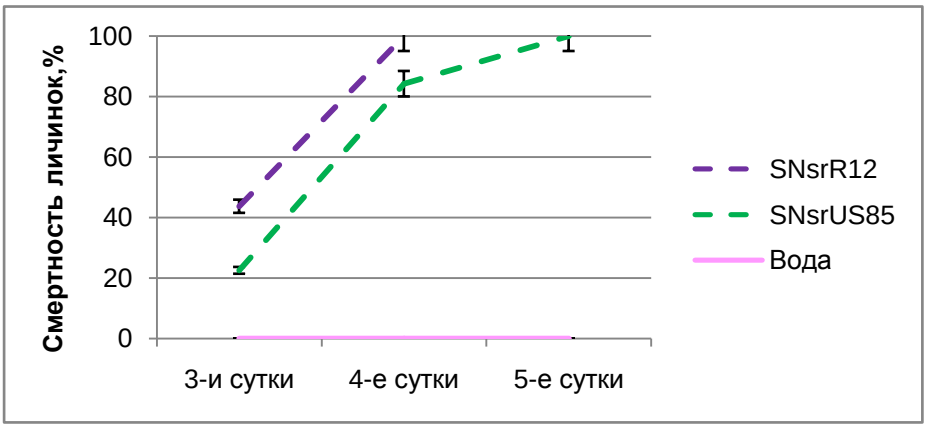
Pic. 18. RPS 4 age larvae mortality in the Orenburgaskaya region after virus strain spraying in concentration $0,25x10^9$



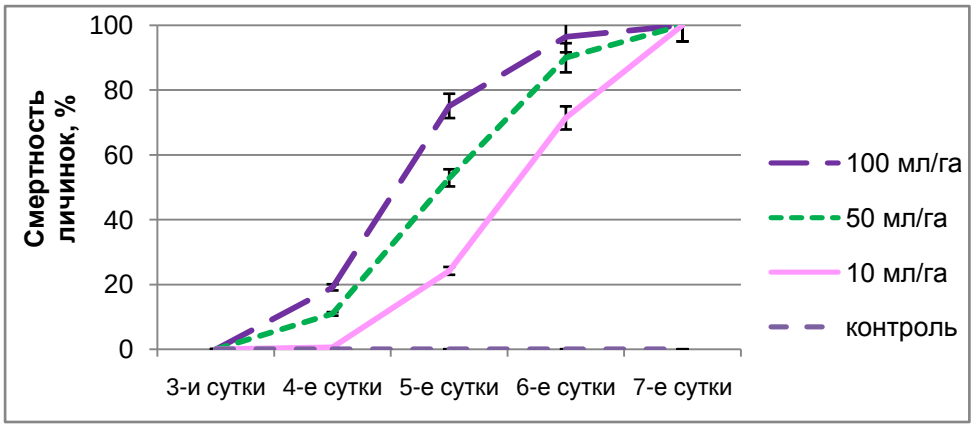
Pic. 19. RPS 1-2 age larvae mortality in the Moscow region after virus strain spraying in concentration $1x10^9$



Pic. 20. RPS 1-2 age larvae mortality in the Moscow region after virus strain spraying in concentration $0,5x10^9$



Pic. 21. RPS 1-2 age larvae mortality in the Moscow region after virus strain spraying in concentration $0,25x10^9$



Pic. 22. RPS 2-3 age larvae mortality in the Moscow region after virus strain spraying in concentration S_{Nsr}R11/12 with various application rates.

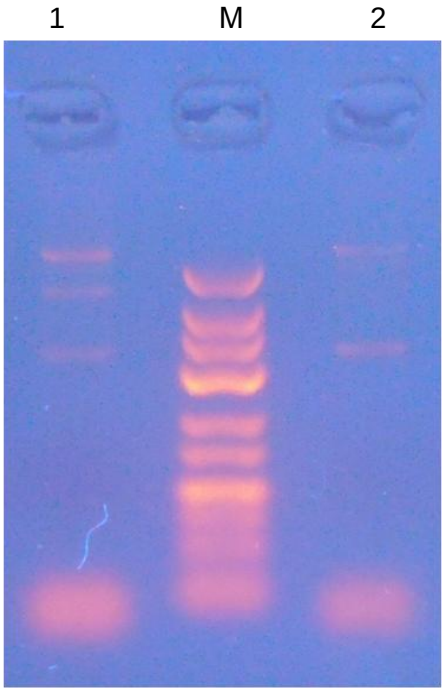
The findings showed that the strain SNsrR11/12 has the greatest virulence with its treatment mortality of main species part starts a day earlier then with SNsrUS85 but final larvae mortality at all concentrations of the two tested strains was simultaneous. Thus the strains selected for small site field experiments showed high efficiency.

Sufficient variation larvae mortality intensity for each strain was found due to suspension concentrations in all options. However total mortality comes on the same day both for minimum and maximum concentrations of the tested strains which points out evolution of virus induced infection process irrespective of concentration. Similar conclusions were based on the results of lab work in the previous research stage.

Comparison of strains at maximum concentrations, 100 times higher than applied earlier by the Virin Diprion developers, of application rate per ha showed that the strain SNsrR11/12 has greater efficiency. It is reasonable to apply the strain SNsrR11/12 in industrial treatments.

The findings imply that treatments of 1-2 age larvae enable fast forest protection effect. Spraying of older age larvae extends its mortality period prolongs needle eating so preparation application rate growth is needed to achieve treatment efficiency.

Experimental treatment results of RPS 2-3 age larvae with the strain SNsrR11/12 showed that larvae mortality started on the 4th day after treatment. At minimum application rate mortality was individual and it increased sufficiently with higher concentrations (pic. 23).



Pic. 23. Electrophoreogram of with the primer olig 25 (GTAATCGACG):
1 – strain S_{Nsr}US85, 2 – strain S_{Nsr}R11/12, M – marker of molecular balance for DNA in length 25, 50, 75, 100, 200, 250, 300, 400, 500, 700 base pair from bottom upwards (Fermentas, Latvia)

There was no mortality in control. On the 5th day after treatment the larvae in control molted into 4 age. There was no any molt in all experimental treatment concentrations, complete feeding termination at maximum concentration was observed on the 4th day after virus introduction, at minimum –on the 5th.

Species dying intensity is higher at maximum application rate in early periods after treatment however final mortality comes simultaneously. Total mortality of all larvae happened on the 7th day. Data statistical processing showed accuracy of variation between the tested strains.

Thus preparation application rate growth surely enables fast treatment efficiency but it is unreasonable to raise active matter consumption rate over 50 ml/ha.

The findings in red pine sawfly mass outbreaks in various outbreak stages show that RSP sensitivity both in population growth and decline was similar and depended more on larvae age.

In the process of lab and field operations the strains SNsrR11/12 (produced from native virus infection from the larvae collected in the Rostovskaya region) and SNsrUS85 (produced from the US produced preparation stored in lab for 27 years) were close both in efficiency and productivity. Such long virus storage period without any special conditions as well as the fact that primary passaging of this strain was done on the Rostovskaya population larvae could result in emergence of a native virus in this strain bio-material induced a need to compare these strains at genetic level.

Polymerase chain reaction was conducted and electrophorerogram of the abovementioned strain DNA amplification products produced to define accuracy of deviations between strains. This work was done by V.Oberemkom researcher of the Tavrichesky University (Simferopol, Crimea).

Polymerase chain reaction (PCR) design was as follows. The reaction mixture with the volume 29 mcl comprised of: 5XPCR-buffer – 5 mcl; MgSO4, 50 мМ – 1,5 mcl; H2O MilliQ – 3 ncl; дНТФ-mix, 2 мМ – 2,5 mcl; Taq-polymerase, 5 ea/mcl – 0,5 mcl; mineral oil – 10,5 mcl; primer olig 25 (100 pmol/mcl) – 1 мкл; TE-buffer with the tested DNA – 5 mcl. PCR mode was as follows: 940C denaturation – 1 min., 400C annealing – 1 min., 720C synthesis – 1 min. – 5 cycles; 940C denaturation – 0,5 min., 400C annealing – 0,5 min., 720C synthesis – 0,5 min. – 40cycles. Sybthesis terminal stage was conducted at 720C – 3 min.

DNA amplification products were separated in 1,8% agar gel and after treatment with ethidium bromide exposed to ultraviolet light (Pic. 26).

The conducted studies resulted in finding of the most RPS larvae nuclear polyhedrosis efficient strain. The strain S_{Nsr}R11/12 was deposited at the State virus collection of the Ivanovsky Virology Research Institute under the Russian Health Ministry. The Certificate of September 20, 2013 has been received, assigned number is 2763.

The selected strain testing enabled its recommendation as a new virus bio-preparation producer against RPS.

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CHARACTERIZATION OF A CHITINASE FROM *APOCHEIMA CINERARIUS* NUCLEOPOLYHEDROVIRUS

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摘要：杆状病毒已成为生物防治应用中不可缺少杀虫剂。文中通过在大肠杆菌中超表达春尺蠖核型多角体病毒（ApciNPV）中的几丁质酶，利用亲和色谱技术纯化获得具有生物活性的几丁质酶蛋白。三维结构显示ApciNPV几丁质酶具有与几丁质底物结合的N端多囊肾1(PKD1)结构域和18家族糖水解酶特有的α/β TIM 桶状催化区域。酶活分析表明，ApciNPV几丁质酶蛋白具有内源和外源几丁质酶的活性。令人感兴趣的是，ApciNPV几丁质酶蛋白对甜菜夜蛾、美国白蛾、棉铃虫和舞毒蛾具有强的杀虫活性。该研究结果为害虫的生物防治提供候选蛋白。

关键词：春尺蠖核型多角体病毒，几丁质酶，重组蛋白，杀虫活性

ХАРАКТЕРИСТИКА ХИТИНАЗЫ ВИРУСА ЯДЕРНОГО ПОЛИЭДРОЗА APOCHEIMA CINERARIUS

Бакуловирусы являются важными агентами биологической защиты растений от вредных насекомых. В данном исследовании изучена хитиназа вируса ядерного полиэдроза *Apocheima cinerarius*. Её экспрессировали до высоких уровней в *Escherichia coli* и очищали аффинной хроматографией. Прогнозирование трехмерной структуры хитиназы ApciNPV показало, что белок имеет N-концевую поликистозную почку 1 (PKD1) домен для подавления хитинового субстрата и каталитический домен α / β TIM bar, характерный для гликогидразы семейства 18. Анализ активности ферментов показал, что хитиназа ApciNPV обладает как эндо-, так и экзохитиназной активностью. Интересно, что хитиназа ApciNPV проявила сильную инсектицидную активность против *Apocheima cinerarius, Spodoptera exigua, Hyphantria cunea, Helicoverpa armigera* и *Lymantria dispar*. Результаты показывают, что хитиназа ApciNPV является хорошим белком-кандидатом для значительного вклада в борьбу с вредителями.

Ключевые слова: вирус ядерного полиэдроза Apocheima cinerarius, хитиназа, рекомбинантный белок, инсектицидная активность.

ABSTRACT:

Baculoviruses are important biological control agents against insect pests. In this work, a chitinase from *Apocheima cinerarius* nucleopolyhedrovirus (ApciNPV) was expressed to high levels in *Escherichia coli* and purified by affinity chromatography. The three-dimensional structure prediction of ApciNPV chitinase indicated the protein has an N-terminal polycystic kidney 1(PKD1) domain for chitin-substrate feeding and α/β TIM barrel catalytic domain characteristic of a family 18 glycohydrolase. Enzyme activity analysis showed that ApciNPV chitinase had both endo- and exo-chitinase activities. Interestingly, the ApciNPV chitinase displayed a strong insecticidal activity against *Apocheima cinerarius*, *Spodoptera exigua*, *Hyphantria cunea*, *Helicoverpa armigera* and *Lymantria dispar*. The results suggest that ApciNPV chitinase is a good candidate protein for significantly contributing to pest control.

Key words: *Apocheima cinerarius* nucleopolyhedrovirus; Chitinase; Recombinant protein; Insecticidal activity

Introduction

In insects, chitin is the major polysaccharide present in the insect cuticle, gut lining or peritrophic matrix, salivary gland, trachea, eggshells and muscle attachment points [1]. Chitinases catalyze the degradation of chitin, usually through hydrolysis of the β-1,4-linkage of the N-acetylglucosamine polymer of chitin to disrupt cuticle and gut physiology in many insect species [2]. Moreover, chitinases also play important roles in morphogenesis and cell division of organisms and inhibiting the growth of fungal mycelium in plants [3]. Chitinases, bioinsecticides and being paid close attention to their exploitation, are thought to be more environment-friendly than chemical pesticide in transgenic plants and biological control agents. Therefore, many chitinases have been isolated from natural sources such as animals, plants and bacteria [4]. Based on their different amino acid sequences, molecular structures and hydrolytic mechanisms, chitinases are classified into two categories: family 18 using the mechanism of substrate-assisted catalysis and family 19 demonstrating acid catalysis [3, 5]. Most of the prokaryotic and eukaryotic chitinases belong to family 18 whereas chitinases of higher plants and some Gram positive bacteria are grouped in family 19 [2]. These two families contain three primary mechanisms in degrading the chitin chain. Endochitinases cleave chitin randomly at internal sites [6], exochitinases cleave off chitobiose $(GlcNAc)_2$ or chitotriose $(GlcNAc)_3$ from the reducing or non-reducing end of the chitin microfibril [7] and N-acetylglucosaminidases, the third class of chitinolytic enzymes release monomers of GlcNAc [8-10].

Many baculovirus chitinases belong to glycosyl hydrolase family 18 [11]. The enzymes retain high endo- and exo-chitinase activities between pH 3.0 and 10.0 and even higher alkaline conditions [12, 13]. Baculovirus chitinases are localized within the endoplasmic reticulum (ER) of infected cells because of the presence of the carboxy-terminal ER retention motif [14-16]. The motif is probably involved in hindering the secretory pathway of chitinase and redistribution of chitinase within the cell during virus infection [12]. Deletion and mutation of the Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) chitinase KDEL motif resulted in the extracellular release of

chitinase and promoting the liquefaction of these insect larvae [15, 16]. The new recombinant AcMNPV with KDEL-deficient chitinase enhanced the insecticidal activity against Trichoplusia ni larvae and reduced the lethal dose and lethal time associated with infection [16].

Baculovirus chitinases are considered responsible for the final liquefaction of infected host larvae [12, 13] and are used as a new tool for insect control. For example, the ChiA protein from AcMNPV indicated 100% mortality for Bombyx mori larvae and resulted in a significant increase of perforations on the peritrophic membrane (PM) in number and in size [17]. The deletion of chiA from B. mori nucleopolyhedrovirus (BmNPV) could delay the cell lysis and decrease the haemolymph turbidity and the degradation of the body in silkworm larvae [18]. Furthermore, chitinase and V-cathepsin, a cysteine protease for the degradation of the proteinaceous components of cadavers could together promote the liquefaction of the host after death [19, 20]. Deletions of the chiA and cathepsin genes of AcMNPV abrogated the liquefaction process [13, 21].

Apocheima cinerarius nucleopolyhedrovirus (ApciNPV) was first discovered and identified by the Institute of Forest Ecology and Environment Conservation, Chinese Academy of Forestry in 1979 [22, 23], belonging to the Baculoviridae family, Alphabaculovirus genus. The virus exhibited high virulence against the larvae of A. cinerarius [24]. Although the ApciNPV has become an important biological insecticide which is now commercially available in China [24], the insecticidal genes from the ApciNPV genome have huge potential in biocontrol and transgenic engineering against other insects.

In this paper, we report the characterization of an insecticidal protein-chitinase from ApciNPV. We also showed the expression of the chitinase in Escherichia coli and demonstrated insecticidal activity against A. cinerarius, Spodoptera exigua, Hyphantria cunea, Helicoverpa armigera and Lymantria dispar. The results suggest that the ApciNPV chitinase can significantly contribute to its potential use as a new tool for pest control.

Materials and Methods

Viruses

The ApciNPV was provided by the Research Centre of Forest Insect Virus, Chinese Academy of Forestry. Virus occlusion bodies (OBs) were purified by centrifugation in a sucrose gradient of 40-60% (w/w) at 10,000 ×g for 30 min at room temperature. The bands containing the virus were collected and washed with sterile water, then centrifuged at 13,200 ×g for 30 min at 4℃.

Purification of viral DNA

Purified ApciNPV was suspended in buffer consisting of 0.1 M Na_2CO_3, 0.15 M NaCl and 0.05 M EDTA, pH10.8, and incubated at 37℃ for 1 h to dissolve the polyhedron matrix. The pH of the suspension was adjusted to 7.0 with 0.1 M HCl, and then the SDS and proteinase K (100 mg/mL) were added to final concentrations of 0.5% and 50 μg/mL repectivity at 55℃ for 3 h. The solution was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform, respectively. DNA was precipitated with two volumes ethanol at -20℃ for 2 h, pelleted by centrifugation at 13,200 ×g for 10 min. The precipitate was dissolved in TE buffer (pH 8.0) and stored at -20℃.

Construction of bacterial expression plasmids

A truncated sequence of orf38 chitinase gene lacking its C-terminal ER retention motif (HTEL) was amplified from ApciNPV genome (GenBank accession number: FJ914221) using primers P1 (5’ –

AAAAggATCCatgcattggtgcgtcaaccgcga-3' and P2 5'–CCCCCTgCAgTTTTATATTAGATTTATAATGT -3'). BamH Ⅰ and Pst Ⅰ restriction sites (underlined) were added at the 5'end of the P1 and P2 primer, respectively. The P1 primer anneals at position 43794 and P2 at position 42224 of the viral genome and they amplify a 1569 bp product. The PCR solution included 40 ng DNA, 0.1 μmol forward and reverse primers, 0.4 mM dNTP, 5U of Expand High Fidelity Taq polymerase (Roche), 1×polymerase buffer (containing $MgCl_2$) and final volume of 50 μL. The reaction of PCR started at 94℃ for 5 min, and the following cycle was repeated 33 times: 94℃ for 30 s, 60℃ for 45 s, 72℃ for 1 min, and final extension went on at 72℃ for 10 min. The resulting DNA fragment was sub-cloned into the pQE30 vector (Novagen) with 6×his-tag gene under the control of the T5 promoter.

Over-expression and purification of the recombinant protein

The ApciNPV chitinase was over-expressed as a fusion protein with the 6×his-tag in E. coli strain M15 (Novagen). Small-scale (3 mL) cells were cultured with a rotary shaker at 37℃ until OD 600=0.6 and the recombinant protein was induced with 0.2 mM IPTG at 16℃ for 15 h. The cells were harvested by centrifugation, kept at -20℃ for 30 min, and then suspended in FastBreak™ Cell lysis Reagent (Promega). The amount of soluble and insoluble recombinant protein was determined by MagneHis™ Protein Purification System according to the manufacturer's instructions (Promega) and checked by separation on sodium dodecyl sulfate (SDS) 12% polyacrylamide gels. Large-scale recombinant protein was obtained by adding 0.2 mM IPTG to 1 L E. coli log-phase culture (OD 600=0.6). After induction of 15 h at 16℃, the cells were harvested and lysed in 80 mL buffer A (20 mM Tris-HCl, 150 mM NaCl, 10 mM Imidazole, pH 7.5), and then sonicated on ice with Sonifier (300W, 3s/2s). After centrifugation, the soluble fraction was applied to Ni Sepharose 6 Fast Flow (GE healthcare) column. The column was equilibrated with buffer A and initially eluted with buffer B (20 mM Tris-HCl, 150 mM NaCl, 20 mM Imidazole, pH 7.5). Adsorbed protein was eluted with buffer C (20 mM Tris-HCl, 150 mM NaCl, 200 mM Imidazole, pH 7.5) and buffer D (20 mM Tris-HCl, 150 mM NaCl, 500 mM Imidazole, pH 7.5), sequentially. The identification and purity of the samples were confirmed by SDS-PAGE (12% gel).

Enzymatic activity of ApciNPV chitinase produced in E. coli

Enzyme activity of the recombinant ApciNPV chitinase was quantified as previously described [6, 17] using 4-methylumbelliferyl β-D-N,N′ diacetilchitobioside [4MU-$(GluNAC)_2$] and 4- methylumbelliferyl β-D-N, N′,N″ triacetilchitotrioside [4MU-$(GluNAC)_3$] substrates to estimate exo-chitinase and endo-chitinase activities, respectively. For each standard assay, 20 μL McIlvaine's buffer (0.1 M citric acid, 0.2 M dibasic sodium phosphate, pH 5.0) and 5 μL appropriate substrates were mixed and then different amounts of protein were added into each tube. After incubating at 30℃ for 30 min, the reaction was terminated by adding 120 μL of 1 M glycine/NaOH buffer, pH10.6 for 5 min. Fluorescence was detected using a Fluoroskan fluorimeter (Thermo) with an excitation at 360 nm and an emission at 460 nm. All experiments were repeated three times.

Insect bioassays

A. cinerarius, S. exigua, H. cunea, H. armigera and L. dispar were provided by the Research Centre of Forest Insect Virus, Chinese Academy of Forestry. Larvae were fed on artificial diet and reared at 26±1℃, 60-70% relative humidity and 14:10 h light:dark photoperiod. The insecticidal activity

assay in vivo and determination of 50% lethal concentration (LC_{50}) was performed as previously described [25] and partly modified. Briefly, 100 μL volume of each of five appropriate doses (1, 2.5, 4, 5.5 and 7ng/μL) of ApciNPV chitinase solution (concentration in ng) dissolved in elution buffer were applied to the surface of artificial diet in each 2-cm^2 well (Sterilin plates). Control diet was added with elution buffer D only. After the plates dried, the third instar larvae were divided into groups of 20 larvae (3 per well) and used in the experiments. Mortality was monitored daily after 5 days and LC_{50} values were estimated by Probit analysis [26].

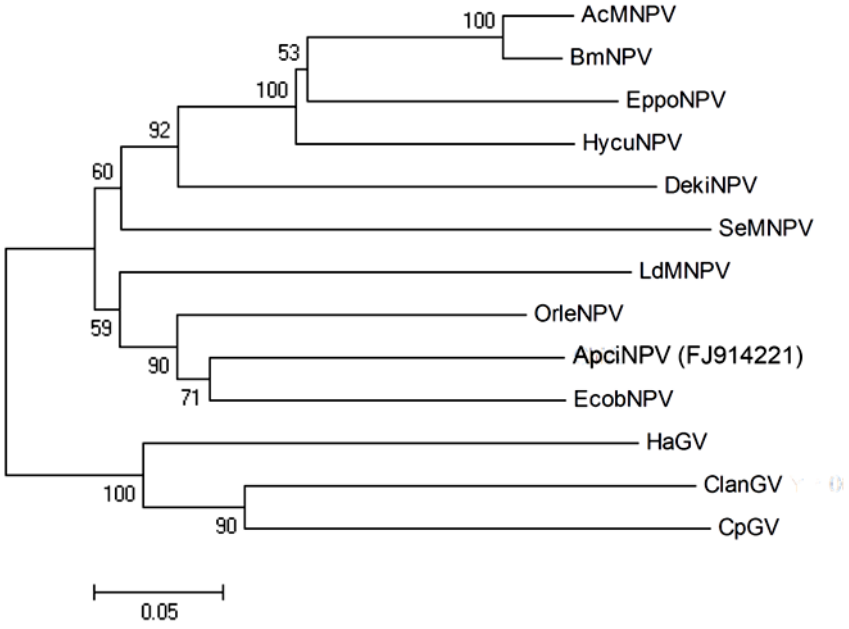
Structural modeling of chitinase from ApciNPV

The three-dimensional structure of ApciNPV chitinase was carried out by the SWISS-MODEL server (http://swissmodel.expasy.org/), and structure template was chitinase of S. marcescens whose PDB code is 1CTN [27].

Results

Analysis of chitinase gene of ApciNPV

The 1581bp orf38 gene from ApciNPV encoded a putative chitinase with 72% amino acid identity to Ectropis obliqua nucleopolyhedrovirus (EcobNPV), 69% identity to Hyphantria cunea nucleopolyhedrovirus (HycuNPV), 65% identity to AcMNPV and 62% identity to S. marcescens. Phylogenetic analyses of baculovirus chitinases consisted of two main branches (Pic. 1), in which ApciNPV chitinase belonged to Alphabaculovirus genus with EcobNPV (YP_874243), Orgyia leucostigma nucleopolyhedrovirus (OrleNPV, YP_001650934), Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV, NP_047707), AcMNPV (NP_054156), BmNPV (NP_047523), Epiphyas postvittana nucleopolyhedrovirus (EppoNPV, NP_203279) HycuNPV

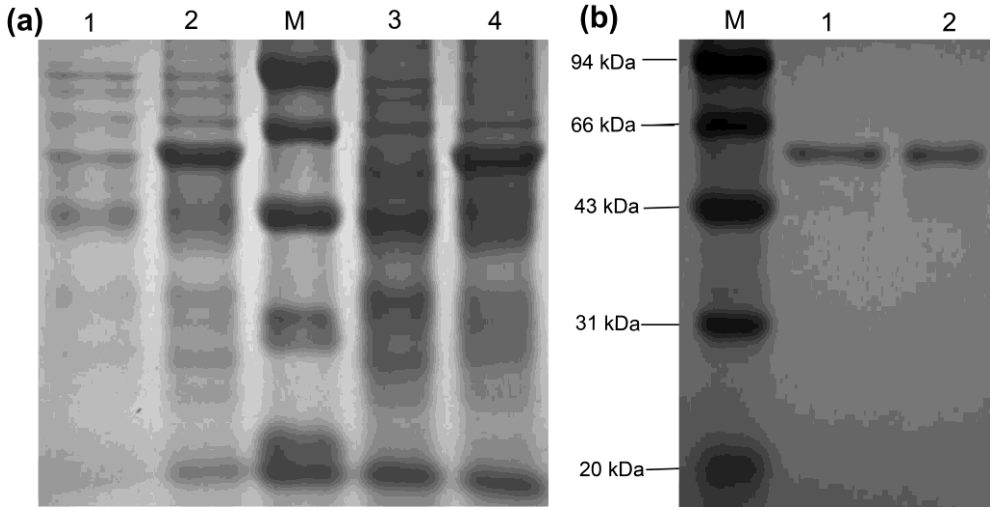


Pic. 1 Phylogenetic tree of chitinases from baculovirus. The tree was constructed by the neighbor-joining method and bootstrap values are indicated at the branches. Amino acid sequences of chitinases come from ApciNPV (FJ914221), AcMNPV (NP_054156), BmNPV (NP_047523), EppoNPV (NP_203279), HycuNPV (YP_473218), DekiNPV (AFP66961), SeMNPV (NP_046280), LdMNPV (NP_047707), OrleNPV (YP_001650934), EcobNPV (YP_874243), HaGV (YP_001649087), ClanGV (YP_004376217) and CpGV (YP_148794).

(YP_473218), Dendrolimus kikuchii nucleopolyhedrovirus (DekiNPV, AFP66961) and Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV, NP_046280). This suggested that they may have similar features and functions. Furthermore, the ApciNPV chitinase contained a predicted polycystic kidney (PKD1) domain at 13-93 aa and a characteristic endoplasmic reticulum targeting sequence, HTEL at C-terminus. The analysis of ApciNPV chitinase functional motif revealed a conserved family 18 glycohydrolase motif (SIGGWT) and the consensus Prosite motif (FDGVDIDWE) containing the critical glutamate residue which may act as a proton donor in the catalytic domain [28, 29].

Expression and purification of native ApciNPV chitinase

A truncated form of the ApciNVP chitinase gene, encoding for 523 residues protein with a theoretical molecular mass of 58.2 kDa, was cloned from the genome of ApciNPV by PCR. The sequence was amplified according to the primers from the ApciNPV genome (GenBank accession number: FJ914221). The PCR product was cloned into the pQE30 vector and then over-expressed in E. coli with the N-terminal 6×His-tag. The recombinant protein was found in the cellular soluble fraction by a feasible condition with 0.2 mM IPTG at 16℃ (Pic. 2a, Lane 2). The recombinant His-tagged chitinase was purified using Ni^{2+}– affinity chromatography and assessed by SDS-PAGE analysis from a large-scale expression cultures (Pic. 2b, Lane 1 and 2).

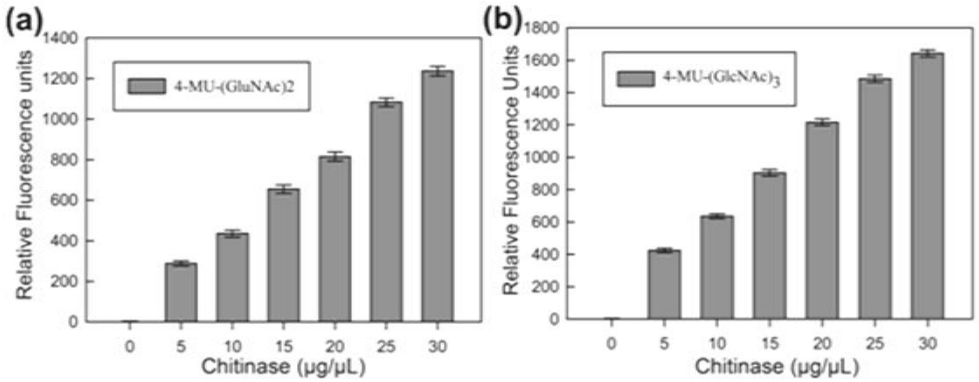


Pic. 2. SDS-PAGE analysis for expression and Ni^{2+}-affinity chromatography of the ApciNPV chitinase. (**a**) Expression of the recombinant ApciNPV chitinase from small-scale by IPTG induced bacterial cultures. Lane 1, the insoluble fraction of induced cells after sonication; Lane 2, the soluble fraction of induced cells after sonication; Lane M, molecular mass markers (from top to down 94.0, 66.2, 43.0, 31.0, and 20.0 kDa); Lane 4, culture pellet (uninduced); Lane 5, culture pellet (induced with 0.2 mM IPTG at 16℃). (**b**) Purification by affinity chromatography under native condition of soluble proteins from large-scale IPTG-induced bacterial cultures. 10 µL samples containing 3 µg of protein were loaded to each lane. Lane 1 indicates the fractions eluted by 20 mM Tris, 150 mM NaCl, 200 mM Imidazole, pH 7.5. Lane 2 displays the fractions eluted by 500 mM Imidazole.

Enzyme activity analysis of ApciNPV chitinase

In order to test chitinases exo- and endo-chitinase activities, we performed the enzyme activity using 4-MU-$(GlcNAC)_2$ and 4-MU-$(GlcNAC)_3$

substrates, respectively. As shown in pic. 3, both exo- and endo-chitinase activities increased linearly with the ApciNPV chitinase concentration up to 4.3 fold and 5.2 fold respectively, over their lowest levels. These data indicated that ApciNPV chitinase produced in E. coli was active and exhibited its native exo- and endo-chitinolytic activities.



Pic. 3. Enzymatic assay of the ApciNPV chitinases from E. coli cells. Increasing amounts of the the recombinant chitinase were incubated either with 4-MU-$(GlcNAc)_2$ to detect exo-chitinase activity (**a**) with 4-MU-$(GlcNAc)_3$ to detect endo-chitinase activity (**b**). Results are expressed as means±SE (n=3).

In vivo assays on larvae

The ApciNPV chitinase showed an obvious insecticidal activity against A. cinerarius, S. exigua, H. cunea, H. armigera and L. dispar. The LC_{50} (50% lethal concentration) values of the larvae were 175.6, 178.5, 279.1, 325.4 and 382.7 ng/cm^2, respectively (Table 1).

Table 1. **Insecticidal activities of ApciNPV chitinase**

Insect species	LC_{50} (ng/cm^2)[a]	Fl 95min-max[b]	Slope
Apocheima cinerarius	175.6	73.5-543.2	2.4
Spodoptera exigua	178.5	64.2-574.5	1.8
Hyphantria cunea	279.1	87.5-628.4	3.1
Helicoverp armigera	325.4	84.3-419.7	2.8
Lymantria dispar	382.7	97.5-643.6	1.5

a LC_{50}, 50% lethal concentration.
b Fl 95min-max, 95% confidence limit.

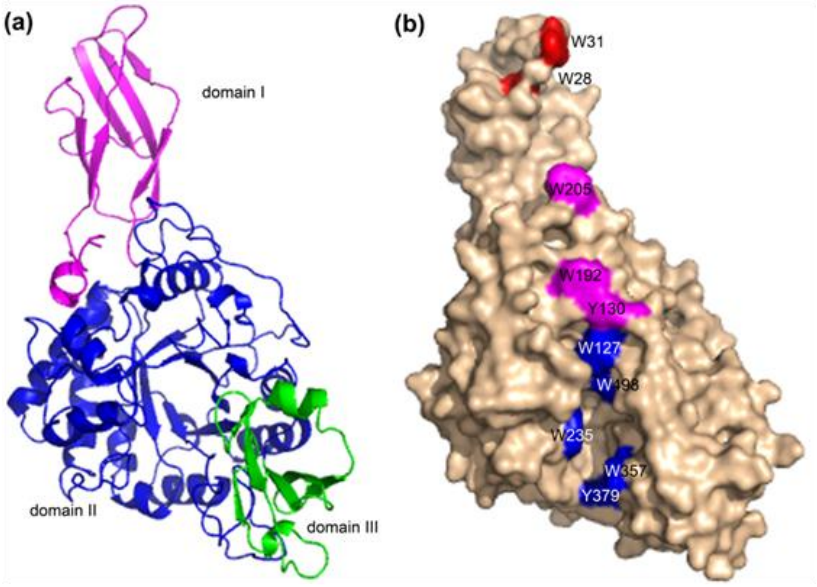
Analysis of the predicted three-dimensional structure of ApciNPVchitinase

To understand the mechanism of ApciNPV chitinase insecticidal activity, we predicted the three-dimensional structure by computer modeling (Pic. 4).

The model was built using the SmChiA from *S. marcescens* which had 62% sequence identity with chitinase from ApciNPV by SWISS-MODEL server. The ApciNPV chitinase model had three structural domains (Pic. 4a), domain Ⅰ was built a polycystic kidney 1(PKD1) domain formed an immunoglobulin-like fold; domain Ⅱ formed an eight-stranded α/β TIM barrel, comprising residues 94-404 and 474-517 and domain Ⅲ was composed of five antiparallel β-strands, one of which was interrupted. Furthermore, the conserved aromatic residues of ApciNPV chitinase in the chitin-feeding mechanism and substrate binding were

deduced on the surface of the protein in positions similar to SmChiA from *S. marcescens* and other chitinases [28, 29]. These aromatic residues included W28 and W31 along the immunoglobulin-like fold, W192, W205 and Y130 leading into the catalytic cleft and W127, W498, W235, Y379 and W357 forming the catalytic binding site (Pic. 4b). It suggests that the predicted aromatic residues from ApciNPV chitinase may be an essential feature for the observed insecticidal activity.

Discussion



Pic. 4. Predicted three-dimensional structure of ApciNPV chitinase. (**a**) A ribbon diagram of ApciNPV chitinase showing the three domains in magenta (Ⅰ), blue (Ⅱ) and green (Ⅲ). (**b**) Representation of the molecular surface of ApciNPV chitinase. The conserved aromatic residues of immunoglobulin-like fold are shown red. The catalytic cleft is colored magenta. The catalytic binding-site is displayed in blue.

In this paper, the chitinase from *Apocheima cinerarius* nucleopolyhedrovirus was characterized. The ApciNPV chitinase showed high levels of sequences identity to the chitinase of AcMNPV, CpGV and *S. marcescens* (Pic. S1). They revealed a conserved family 18 glycohydrolase motif (SIGGWT) and the consensus Prosite motif (FDGVDIDWE). It proved to be a canonical baculovirus chitinases with almost all properties being essentially similar to those of the AcMNPV, and suggested that the ApciNPV chitinase is closely related to the chitinase of *S. marcescens*. The ApciNPV chitinase contained a C-terminal ER-retention sequence (HTEL) that is probably involved in retaining the enzyme inside the cell. ER-retention sequences have also been identified in the chitinases of *B. mori* NPV [30], AcMNPV [15, 16] and HaSNPV [11]. Because of the ER retention motif (KDEL), chitinase of AcMNPV was localized in the ER during infection, and deletion of KDEL motif resulted in earlier secretion into the media from infected cells [15, 16]. Furthermore, modeling of the ApciNPV chitinase against the known structure of SmchiA [31] identified two major domains: a PKD1 domain and a catalytic domain indicative of the family 18 glycohydrolase (Pic. 4) . The

PKD1 domain formed an immunoglobulin-like fold was involved in carbohydrate splitting and guiding the substrate into the catalytic groove [32, 33]. The catalytic domain produced a deep substrate-binding cleft [28, 29]. In addition, conserved tryptophan residues along the PKD1 fold and other aromatic residues in the catalytic domain were found on the surface of SmChiA and other chitinases [28, 29]. The conserved residues of SmChiA, included W69 and W33 in the N-terminal domain and W245 in the catalytic domain, which play a vital role in the chitin binding, and F-232 guides the chitin chain into the catalytic cleft [28]. Young et al. reported ORF110 from *Epiphyas postvittana* nucleopolyhedrovirus (EppoNPV) has conserved residues W223, W236 and Y161 leading into the catalytic cleft and W158, W529, W266, Y410 and W388 forming the catalytic binding site [29]. Analogously, ApciNPV chitinase has W28 and W31 in the corresponding position along the immunoglobulin-like fold, and W192, W205 and Y130 may also aid in feeding the insoluble chitin chain into the catalytic pocket and thus to the active site, and W127, Y498, W235, Y379 and W357 probably form hydrophobic interactions with hydrophobic faces of the alternating glucosamine units of chitin, thus producing the binding sites.

To obtain the biological activity, the truncated ApciNPV chitinase gene lacking the C-terminal ER-retention sequence (HTEL) was expressed in *E. coli*. The recombinant ApciNPV chitinase stored as a small amount of inclusion bodies and large amount of soluble cytosolic components (Pic. 2a). In order to avoid the multi-step renaturing processes to recover the enzyme activities, the recombinant protein was efficiently purified in its native form and further identified by SDS-PAGE (Pic. 2b). The ApciNPV chitinase showed both exo- and endo-chitinase activities using either 4-MU-$(GlcNAc)_2$ or 4-MU-$(GlcNAc)_3$ substrate, respectively (Pic. 3). It was consistent with previous reports stating that baculovirus chitinases had both exo- and endo-chitinases activities [12, 13, 17]. Compared to chitinase from DekiNPV [34] and AcMNPV [17], the ApciNPV chitinase were higher exo- and endo-chitinase activities than the DekiNPV chitinase, yet lower exo-chitinase activity than the AcMNPV chitinase. In addition, the chitinase of ApciNPV displayed an obvious insecticidal activity agianst *A. cinerarius*, *S*. *exigua*, *H*. *cunea*, *H*. *armigera* and *L*. *dispar* (Table 1). Compared to chitinase from DekiNPV [34], the LC_{50} values of ApciNPV chitinase were lower than *S*. *exigua*, *H*. *cunea*, *H*. *armigera* and *L*. *dispar*. It indicated that ApciNPV chitinase had a great potential in pest control. Previous studies had also shown the role of baculovirus chitinase. Rao et al. (2004) reported that ChiA of AcMNPV resulted in the decrease of larval body weight (LW) of *B. mori* at a dose of 0.56 μg/g LW and 100% mortality at a dose of 1μg/g LW [17]. ChiA from BmNPV could aid in degrading the chitinous PM lining the *B*. *mori* larval midgut [18]. Whether the insecticidal mechanism of ApciNPV chitinase is due to the damage of PM needs to be investigated further.

In summary, these results presented in this work identified a new chitinase from ApciNPV chitinase as a candidate protein capable of protecting crops and forests against insect pests.

Acknowledgments

We thank Xue Zhang for critical reading and English editing of the manuscript. This work was supported by the Program of National Nature Science Foundation of China (grant no. 31200491) and State 863 Project funded by Ministry of Science and Technology (grant no. 2012AA101503) P.R. China.

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CHARACTERIZATION OF A NOVEL CHITINASE DKCHI FROM *DENDROLIMUS KIKUCHII* NUCLEOPOLYHEDROVIRUS

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摘要：思茅松毛虫核型多角体病毒（DekiNPV）是一种新型的、具有生防价值的杆状病毒。文中通过构建思茅松毛虫NPV病毒的DNA文库，获得了大小为1755 bp的几丁质酶基因。该基因-
DkChi，编码558aa，分子量为61.6kDa。在IPTG的诱导下，DkChi基因在大肠杆菌中高效表达。通过亲和色谱和生物质谱分别纯化和鉴定了该蛋白。酶活分析表明，DkChi蛋白具有内源和外源几丁质酶的活性。此外，生物活性测定发现，DkChi蛋白对甜菜夜蛾、美国白蛾、棉铃虫和舞毒蛾具有强的杀虫活性。该研究结果为害虫的生物防治提供候选蛋白。

关键词：思茅松毛虫核型多角体病毒，几丁质酶，重组蛋白，生物杀虫剂

ХАРАКТЕРИСТИКА НОВОЙ ХИТИНАЗЫ ВИРУСА ЯДЕРНОГО ПОЛИЭДРОЗА КОКОНОПРЯДА *DENDROLIMUS KIKUCHII*

Вирус ядерного полиэдроза коконопряда *Dendrolimus kikuchii* Matsumura (DkNPV) представляет собой новый нуклеополиэфировирусный штамм, который обладает высоким потенциалом в качестве агента биологической борьбы против *D. kikuchii*. В этой работе геном DkChi 1755 п.о. с гомологией последовательности с геном хитиназы клонировали из геномной ДНК DkNPV с использованием библиотеки фрагментов ДНК. Ген DkChi, кодирующий 558 остатков белка с предсказанной массой 61,6 кДа, экспрессировали на высоких уровнях в *Escherichia coli* и очищали аффинной хроматографией. Результаты методом масс-спектрометрии подтвердили, что полученный белок представлял собой белок DkChi. Анализ активности ферментов показал, что DkChi обладает как эндо-, так и экзо-хитиназной активностью. Интересно, что белок DkChi проявил сильную инсектицидную активность против *Spodoptera exigua, Hyphantria cunea, Helicoverpa armigera* и *Lymantria dispar*. Результаты показывают, что DkChi является хорошим белком-кандидатом для значительного вклада в борьбу с вредителями.

Ключевые слова: вирус ядерного полиэдроза *Dendrolimus kikuchii*, хитиназа, рекомбинантный белок, биоинсектициды.

ABSTRACT:

Dendrolimus kikuchii Matsumura nucleopolyhedrovirus (DkNPV) is a novel nucleopolyhedrovirus strain that has exhibited high potential as biological control agent against *D. kikuchii*. In this work, a 1755-bp *DkChi* gene with sequence homology to chitinase gene was cloned from the genomic DNA of DkNPV using a DNA fragment library. The *DkChi* gene encoding 558 residues protein with a predicted mass of 61.6 kDa was expressed at high levels in *Escherichia coli* and purified by affinity chromatography. We confirmed that the prepared protein was the DkChi protein by mass spectrometry analysis. Enzyme activity analysis showed that DkChi had both endo- and exo-chitinase activities. Interestingly, the DkChi protein displayed a strong insecticidal activity against *Spodoptera exigua*, *Hyphantria cunea*, *Helicoverpa armigera* and *Lymantria dispar*. The results suggest that DkChi is a good candidate protein for significantly contributing to pest control.

Key words: *Dendrolimus kikuchii* nucleopolyhedrovirus; Chitinase; Recombinant protein; Bioinsecticides

Introduction

In insects, chitin is the major polysaccharide present in the insect cuticle, gut lining or peritrophic matrix, salivary gland, trachea, eggshells and muscle attachment points [1]. Chitinases catalyze the degradation of chitin, usually through hydrolysis of the β-1,4-linkage of the N-acetylglucosamine polymer of chitin to disrupt cuticle and gut physiology in many insect species [2]. Moreover, chitinases also play important roles in morphogenesis and cell division of organisms and inhibiting the growth of fungal mycelium in plants [3]. Chitinases, being bioinsecticides paid close attention on their exploitation, are thought to be more environment-friendly than chemical pesticide in transgenic plants and biological control agents. Therefore, many chitinases have been isolated from natural sources such as animals, plants and bacteria. Based on their different amino acid sequences, molecular structures and hydrolytic mechanisms, chitinases are classified into two categories: family 18 using the mechanism of substrate-assisted catalysis and family 19 demonstrating acid catalysis [3, 4]. Most of the prokaryotic and eukaryotic chitinases belong to family 18 whereas chitinases of higher plants and some Gram positive bacteria are grouped in family 19 [2]. These two families contain three primary mechanisms in degrading the chitin chain. Endochitinases cleave chitin randomly at internal sites [5], exochitinases cleave off chitobiose $(GlcNAc)_2$ or chitotriose $(GlcNAc)_3$ from the reducing or non-reducing end of the chitin microfibril [6] and N-acetylglucosaminidases, the third class of chitinolytic enzymes release monomers of GlcNAc [7-9].

Many baculovirus chitinases belong to glycosyl hydrolase family 18 [10]. The enzymes retain high endo- and exo-chitinase activities between pH 3.0 and 10.0 and even higher alkaline conditions [11, 12]. Chitinases in baculoviridae are localized within the endoplasmic reticulum (ER) of infected cells because of the presence of the carboxy-terminal ER retention motif [13-15]. The motif probably involved in hindering the secretory pathway of chitinase and redistribution of chitinase within the cell during virus infection [11]. Deletion and mutation of the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) chitinase KDEL motif, resulted in the releasing of chitinase into extracellular and promoting the liquefaction of insect [14, 15]. The new

recombinant AcMNPV with KDEL-deficient chitinase enhanced the insecticidal activity against *Trichoplusia ni* larvae and reduced the lethal dose and lethal time associated with infection [15].

Baculovirus chitinases are considered responsible for the final liquefaction of infected host larvae [11, 12] and are used as a new tool for insect control. For example, the ChiA protein from AcMNPV indicated 100% mortality for *Bombyx mori* larvae and resulted into a significant increase of perforations on the peritrophic membrane (PM) in number and in size [16]. The deletion of chiA from *B. mori* nucleopolyhedrovirus (BmNPV) could evidently delay the cell lysis and clear haemolymph and less degradation of the body in silkworm larvae [17]. Furthermore, chitinase and V-cathepsin, a cysteine protease for the degradation of the proteinaceous components of cadavers could together promote the liquefaction of the host after death [18, 19]. Such as, the chiA gene or cathepsin of AcMNPV was deleted, which abrogated the liquefaction process [12, 20].

Dendrolimus kikuchii Matsumura nucleopolyhedrovirus (DkNPV) is a new virus strain recently isolated from dead *D. kikuchii* larvae in Mile county, Yunnan province, China [21]. The virus exhibited higher virulence against the larvae of *D. kikuchii* than *D. Kikuchii* Matsumura Nuclear Polyhedrosis Virus (DKMNPV) previously isolated by Yang [21, 22]. Although the DkNPV showed only insecticidal activity against *D. kikuchii*, the insecticidal genes from the DkNPV genome have huge potential in biocontrol and transgenic engineering against other insects.

In this paper, we report the isolation and characterization of an insecticidal protein-DkChi from DkNPV. We also show the expression of *DkChi* gene in *Escherichia coli* performed insecticidal activity against *Spodoptera exigua*, *Hyphantria cunea*, *Helicoverpa armigera* and *Lymantria dispar*. The results suggest that the DkChi can significantly contribute to its potential use as a new tool for pest control.

Materials and Methods

Viruses

The DkNPV was provided by the Research Centre of Forest Insect Virus, Chinese Academy of Forestry. Virus occlusion bodies (OBs) were purified by centrifugation in the sucrose gradient of 40-60% (w/w) at 10,000 g for 30 min at room temperature. The bands containing the virus were collected and washed with sterile water, then centrifuged at 12000 rpm for 30 min at 4℃ with three replications.

Purification of viral DNA

Purified DkNPV was suspended in the buffer with 0.1 M Na_2CO_3, 0.15 M NaCl and 0.05 M EDTA, pH 10.8, and incubated at 37℃ for 1 h to dissolve the polyhedron matrix. The pH of the suspension was adjusted to 7.0 with 0.1 M HCl, and then 0.5% (w/v) SDS and proteinase K (50 mg/L) were added successively at 55℃ for 3 h. The solution was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform, respectively. DNA was precipitated with two volumes ethanol at -20℃ for 2 h, pelleted by centrifugation at 12000 rpm for 10 min. The precipitate was dissolved in TE buffer (pH 8.0) and stored at -20℃.

Preparation of a DNA library

A DNA fragment library of DkNPV was constructed using shotgun. Purified genomic DNA was sheared by ultrasonication into fragments with an average size of 1200 bp. The ends of each random fragment were repaired

using T4 DNA polymerase (Klenow). According to the manufacturer's protocols, viral DNA fragments were then cloned into pUC19. Ligation products were transformed into *E. coli* XL1-Blue competent cells (Stratagene). Recombinants were picked randomly. DNA templates for sequencing were further prepared using Qiaprep Turbo kits (Qiagen) on Qiagen BioRobot 9600. Positive clones were sequenced at the Beijing Genomics Institute in China.

Sequence and phylogenetic analysis

The detected amino acid sequence was carried out using DNAMAN tool. Sequence features, such as signal peptide, p*I* and molecular mass were evaluated using protein analysis tools (http://expasy.org/tools). The conserved domains and motifs were deduced in PredictProtein. Chitinase sequences were selected from NCBI. The mature protein sequences were aligned with Cluster X version 2.0 and gaps were removed from the alignments. The phylogenetic tree of those alignments was calculated by the neighbor-joining method using MEGA 4 program, and bootstrap values from 1000 replicates indicated at the branches.

Construction of bacterial expression plasmids

A truncated sequence of *DkChi* was amplified by PCR, using primers (5' 5'-AAAAGGATCCTTGCCGGGGACGCCACAAATCGA-3' and 5'-AAAAGAGCTCAACGCGCAACACGACCTCAGA-3') to generate *Bam*HⅠ and *Sac* I restriction sites (underlined) at the 5'end and 3'end. The PCR solution included 40 ng DNA, 0.1 μmol forward and reverse primers, 0.4 mM dNTP, 5U of Expand High Fidelity Taq polymerase (Roche), 1×polymerase buffer (containing $MgCl_2$) and final volume of 50 μL. The reaction of PCR started at 94℃ for 5 min, and following cycle was repeated 33 times: 94℃ for 30 s, 60℃ for 45 s, 72℃ for 1 min, and final extension went on at 72℃ for 10 min. The resulting DNA fragment was sub-cloned into the pQE30 vector (Novagen) with 6×his-tag gene under the control of the T5 promoter.

Over-expression and purification of the recombinant protein

The DkChi protein was over-expressed as a fusion protein with the 6×his-tag in *E. coli* strain M15 (Novagen). 1L cells were cultured with a rotary shaker at 37℃ until OD 600=0.6 and the recombinant DkChi was induced with 0.2 mM IPTG at 16℃ for 15 h. The cells were harvested by centrifugation and homogenized in 80 mL buffer A (20 mM Tris-HCl, 150 mM NaCl, 10 mM Imidazole, pH 7.5), and then sonicated on ice with Sonifier (300W, 3s/2s). After centrifugation, the soluble fraction was applied to Ni Sepharose 6 Fast Flow (GE healthcare) column. The column was equilibrated with buffer A and initially eluted with buffer B (20 mM Tris-HCl, 150 mM NaCl, 20 mM Imidazole, pH 7.5). Adsorbed protein was eluted with buffer C (20 mM Tris-HCl, 150 mM NaCl, 200 mM Imidazole, pH 7.5) and buffer D(20 mM Tris-HCl, 150 mM NaCl, 500 mM Imidazole, pH 7.5), sequentially. The adsorbed protein fractions were pooled and dialysed against buffer E (20 mM Tris-HCl pH 7.5) using 10-kDa Centricon concentrator (Millipore). The dialyzed solution was condensed by freeze drying and loaded onto AKTA FPLC Resource S column (GE Healthcare, NJ, USA). The chromatography was equilibrated with buffer E and adsorbed protein was eluted in linear gradient using NaCl from 0 to 0.2 M in buffer E. Finally, the protein was further purified on AKTA FPLC Superdex 75 HR10/30 (GE Healthcare, NJ, USA) in buffer F (10 mM Tris-HCl, 150 mM NaCl, 2 mM DTT, pH 7.5). The identification and purity of the samples were confirmed by SDS-PAGE (12% gel).

Mass spectrometry analysis

The protein strip was removed from the SDS-PAGE gel with a scalpel, crushed, and destained by washing with 25mM ammonium bicarbonate containing 50% acetonitrile for 1 h. Then, the gel pieces were shrunk to 100% acetonitrile and completely dried before tryptic digestion. The protein was digested with 25 mM NH_4HCO_3 containing 0.01% sequence-grade trypsin (Promega, Madison, WI. USA) at 30℃ for overnight, and then the mixture was sonicated for 10 min and centrifuged. The supernatant was removed and then the peptide fragments were extracted twice with saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 60% acetonitrile and 0.1% trifluoroacetic acid). Sample was spotted onto the MALDI target plate and air-dried before mass spectrometric analysis.

The peptide was identified with MALDI-TOF MS (ReFlex III, Bruker USA) and mass fingerprint spectra was acquired and analysed by the National Center of Biomedical Analysis, Academy of Military medical Sciences, Beijing, China.

Protein identification was performed by Mascot search engine (Matrix science) in the NCBI non-redundant database, and the monoisotopic, mass accuracy, 0.2 Da and missed cleavages, 1 were set.

Enzymatic activity of DkChi produced in *E. coli*

Enzyme activity of the recombinant DkChi protein was quantified as previously described [5, 16] using 4-methylumbelliferyl β-D-N,N′ diacetilchitobioside [4MU-$(GluNAC)_2$] and 4-methylumbelliferyl β-D-N, N′,N″ triacetilchitotrioside [4MU-$(GluNAC)_3$] substrates for the estimating of exo-chitinase and endo-chitinase activities, respectively. For each standard assay, 20 uL McIlvaine's buffer (0.1 M citric acid, 0.2 M dibasic sodium phosphate, pH 5.0) and 5 uL appropriate substrates were mixed and then different amounts of protein were added into each tube. After incubating at 30℃ for 30 min, the reaction was terminated by adding 120 uL of 1M glycine/NaOH buffer, pH10.6 for 5 min. Fluorescence was detected using a Fluoroskan fluorimeter (Thermo) with an excitation at 360 nm and an emission at 460 nm. All experiments were repeated three times.

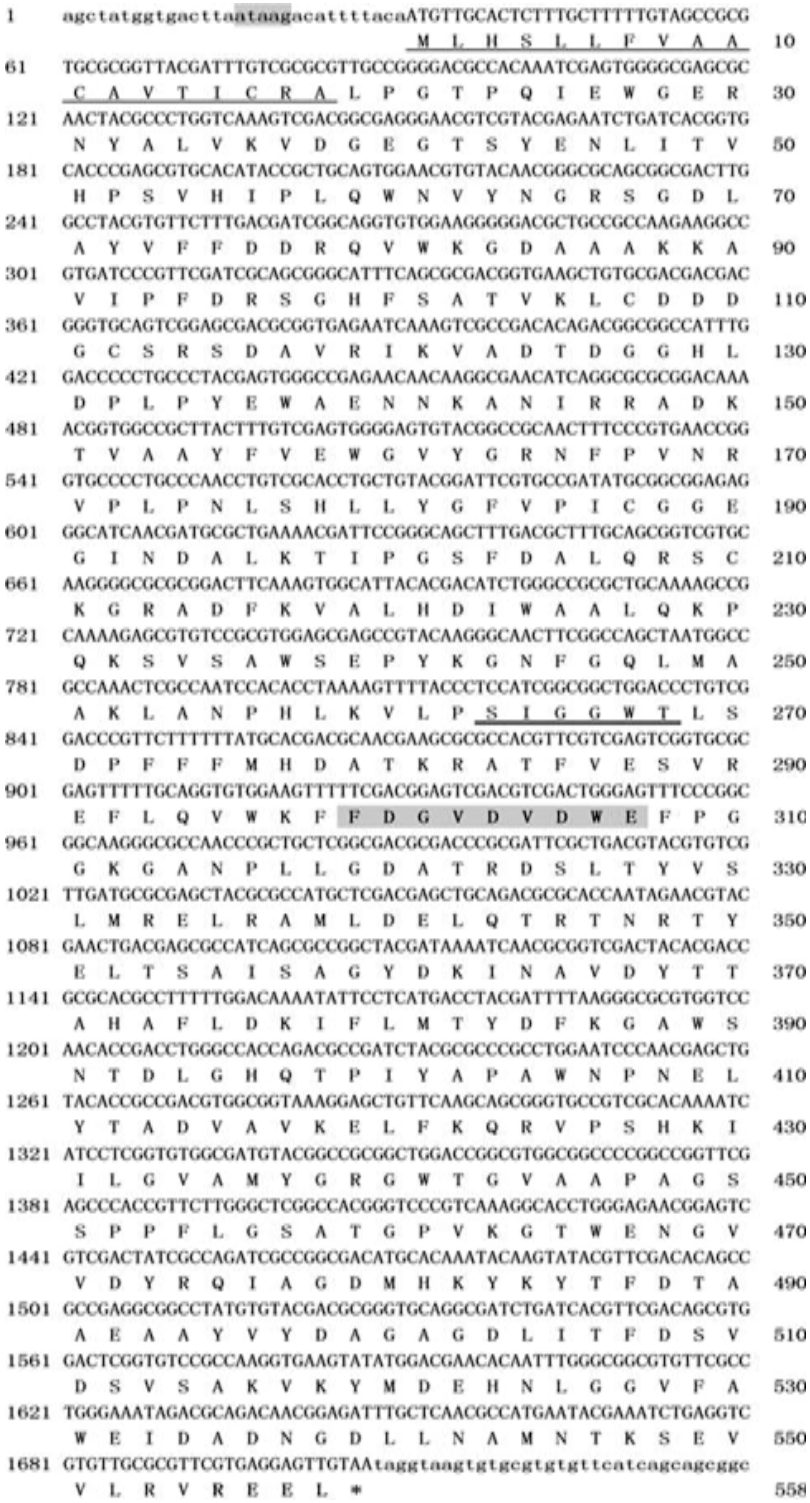
Insect bioassays

S. *exigua*, *H*. *cunea*, *H*. *armigera* and *L*. *dispar* were provided by the Research Centre of Forest Insect Virus, Chinese Academy of Forestry. Larvae were fed on artificial diet and reared at 26±1℃, 60-70% relative humidity and 14:10 h light:dark photoperiod. The insecticidal activity assay in vivo and determination of 50% lethal concentration (LC_{50}) was performed as previously described [23] and partly modified. Briefly, 100 μL volume of each of five appropriate doses of DkChi protein solution (concentration in ng) dissolved in elution buffer were applied to the surface of artificial diet in each 2-cm^2 well (Sterilin plates). Control diet was added with elution buffer F only. After the plates dried, the third instar larvae were divided into groups of 20 larvae (3 per well) and used in the experiments. Mortality was monitored daily after 5 days and LC_{50} values were estimated by Probit analysis [24].

Results

Characterization of *DkChi*

The target DNA was obtained in the DNA fragment library of DkNPV, named DkChi, logged JN680874 on Genebank, shown in Pic. 1.

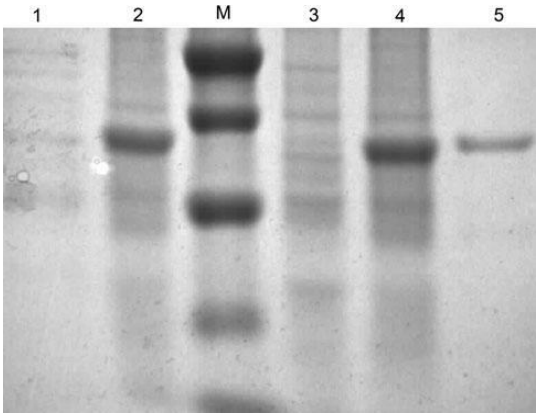


Pic. 1. Nucleotide sequence of *DkChi* and putative amino acid sequence. Capital letters and small letters are denoted as coding region and non-coding region, respectively. Nucleotides are numbered on the left and amino acids are right.* means the termination codon. The signal peptide is underlined. The typical chitinase 18 glycohydrolase motif is marked by double underline. The consensus Prosite motif is displayed with shadow and bold. An endoplasmic reticulum targeting sequence is shown with bold. The putative motif of late transcription initiation is displayed with shadow.

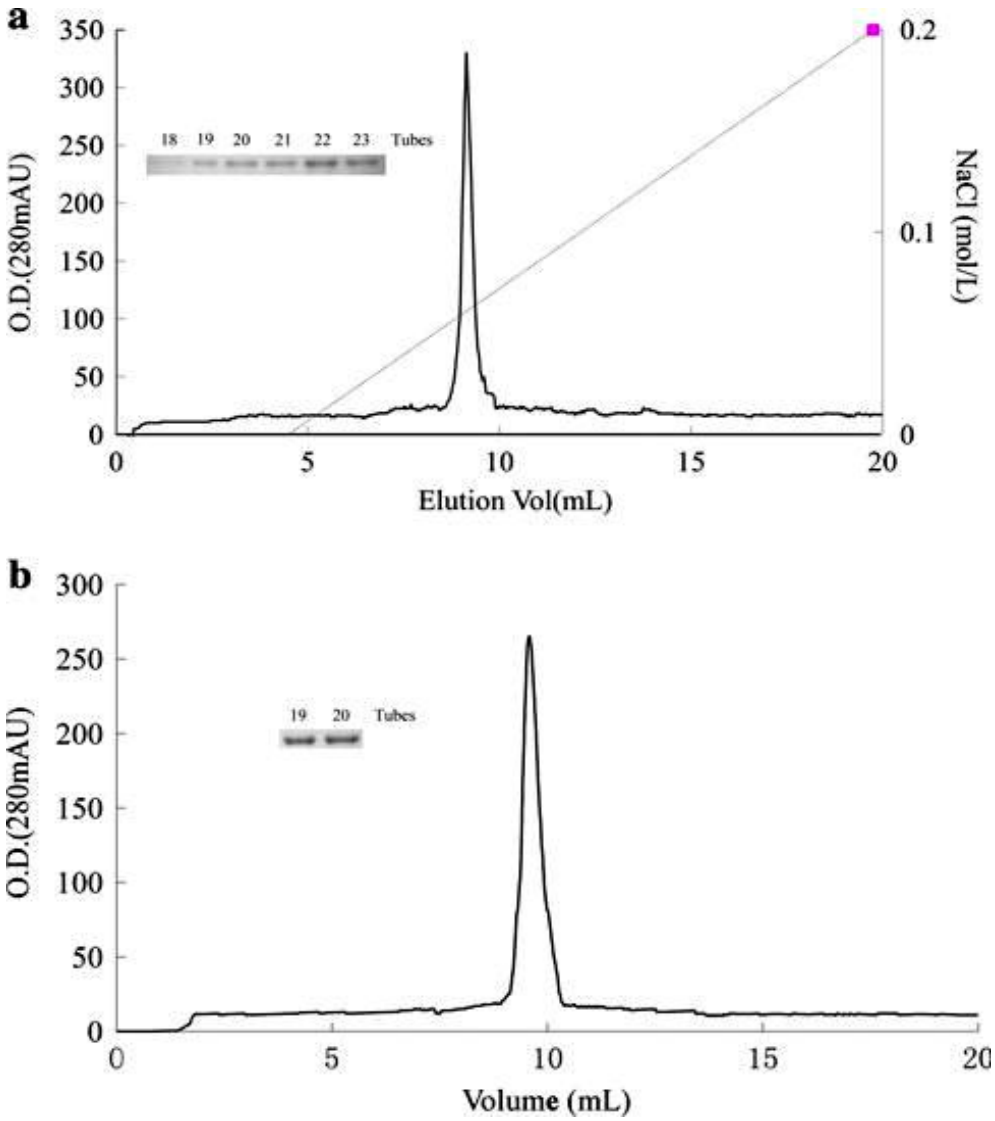
The *CkChn*134 gene was 1755 bp, contained an open reading frame (ORF) with1674 bp, encoding 558 residues protein with a theoretical molecular mass of 61.68 kDa, p*I* 5.7. The deduced protein has a typical feature of signal peptide at 1-18aa. A consensus baculovirus late transcription initiation motif, ATAAG, was found at 16 bp upstream from the putative translation start codon ATG. The typical poly (A) signal, AATAAA was not present downstream of the chitinase ORF. The enzyme contained a predicted polycystic kidney (PKD1) domain at 45-125aa and a characteristic endoplasmic reticulum targeting sequence, REEL at C-terminus. The analysis of DkChi functional motif revealed a conserved family 18 glycohydrolase motif (SIGGWT) and the consensus Prosite motif (FDGIDIDWE) containing the critical glutamate residue which act as a proton donor in the catalytic domain. Furthermore, the conserved aromatic residues of DkChi in the chitin-feeding mechanism and substrate binding were located on the surface of the protein in positions similar to SmChiA from *S*. *marcescens* and other chitinase [25, 26]. These aromatic residues included Trp-27 and Trp-60 along the immunoglobulin-like fold, Trp224, Trp237 and Tyr162 leading into the catalytic cleft and Trp159, Typ531, Trp277, Tyr411 and Trp389 forming the catalytic binding site. Additionally, after analyzing deduced amino acid sequence of DkChi by BLAST in NCBI, we found the DkChi shared high identity with the family 18 chitinases *H. cunea* NPV (70%), *Orgyia pseudotsugata* MNPV (70%) and *A. californica* NPV (69%). Phylogenetic analyses of baculovirus chitinases showed DkChi was grouped with HcNPV, OpMNPV, BmNPV and AcMNPV (Pic. S1). It suggested that they may have similar features and functions.

Expression and purification of the DkChi protein

In order to analysis the biological activity of DkChi, the protein was expressed in *E. coli* with a N-terminal 6×His-tag. The recombinant protein of major soluble fraction was obtained by a feasible condition at 16℃ with 0.2 mM IPTG (Pic. 2, Lane 2). The recombinant His-tagged DkChi was purified using Ni^{2+}– affinity chromatography (Pic. 2, Lane 5). Subsequently, the eluted fractions was collected and further fractionated on a cation exchange chromatography column (Pic. 3a). Finally, the partially purified concentrate protein was further purified through gel filtration chromatography (Pic. 3b).



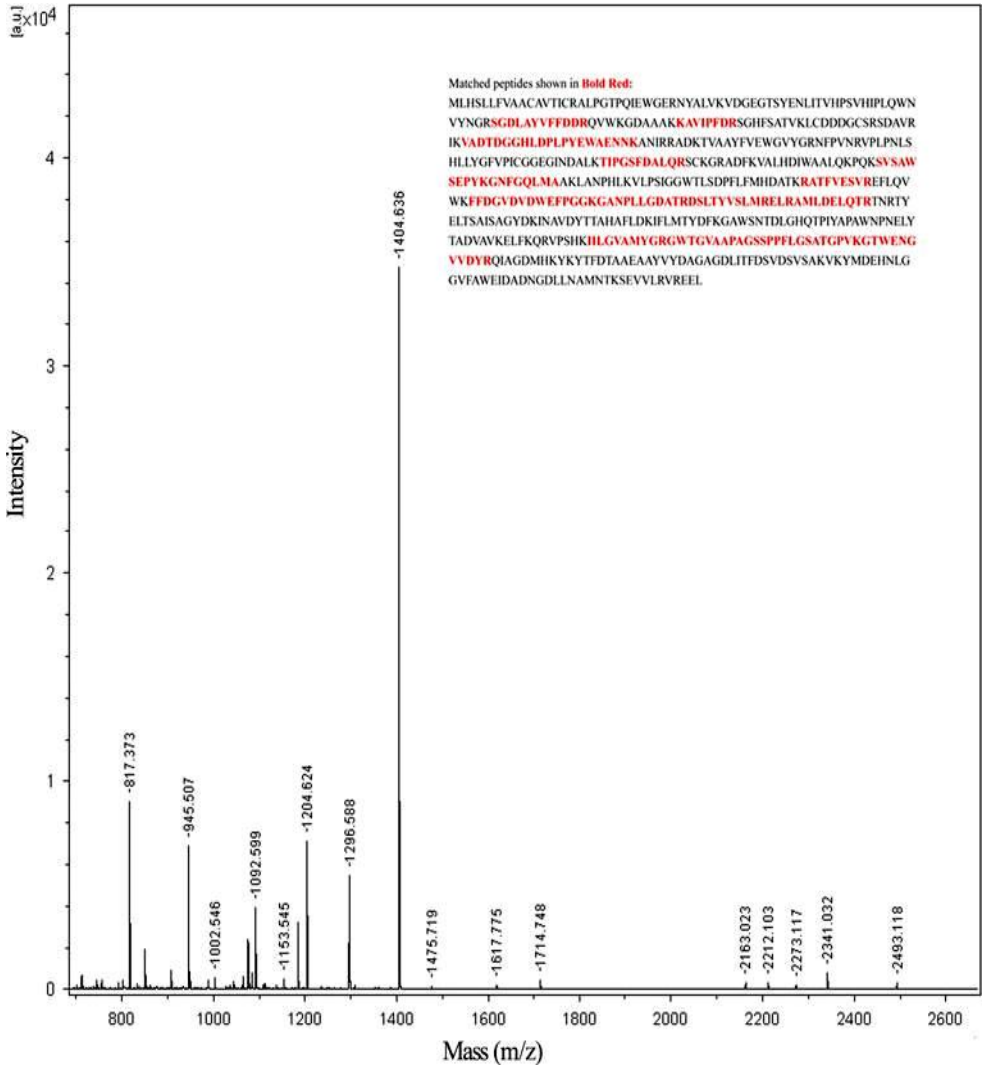
Pic. 2. SDS-PAGE analysis for expression and Ni^{2+}-affinity chromatography of the DkChi protein. Lane 1, the insoluble fraction of induced cells after sonication; Lane 2, the soluble fraction of induced cells after sonication; Lane M, molecular mass markers (from top to down 94.0, 66.2, 43.0, 31.0, and 20.0 KDa); Lane 4, culture pellet (uninduced); Lane 5, culture pellet (induced with 0.2 mM IPTG at 16℃). Lane 6, purified DkChi protein eluted by 20 mM Tris, 150mM NaCl, 500mM Imidazole, pH 7.5.



Pic. 3. Purification of the DkChi protein from *E. coli*. **a** The DkChi protein was purified by cation exchange chromatography on FPLC-Resource S column. **b** The DkChi protein was purification by gel filtration chromatography on FPLC-Superdex 75 HR10/30 column. The purity was checked by SDS-PAGE analysis after each purification procedure.

MALDI-TOF mass spectrometry analysis of the DkChi protein

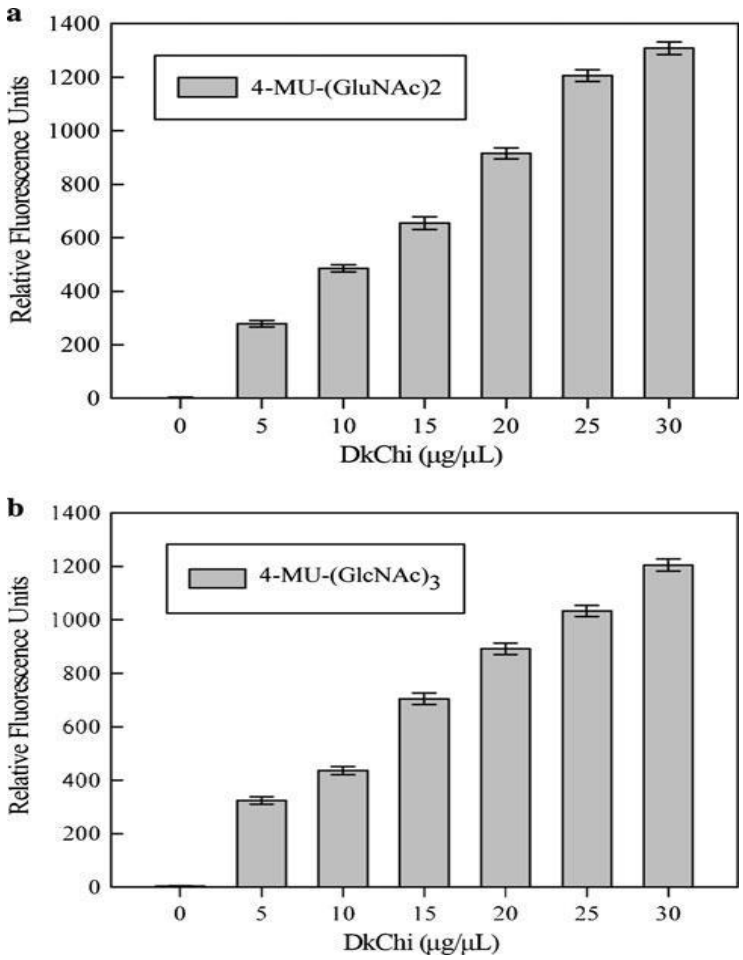
The purified protein was identified by MALDI-TOF-MS on the basis of peptide mass fingerprinting, following in-gel digestion with trypsin. The peptide mass fingerprinting datas were matched with the theoretical peptide masses of all proteins from the viruses of the NCBI database in Mascot. The representative spectrum of the trypsin digestion of protein was showed in pic. 4 and only JN680874 protein from DkNPV was obtained as a result with a score of 123. It demonstrated the purified DkChi protein is the JN680874 protein from DkNPV.



Pic. 4. Identification of the purified DkChi protein using MALDI-TOF peptide mass fingerprint (PMF). The PMF analysis was made from fragments of DkChi protein derived through trypsin digestion. The sequence coverage of these fragments was shown in bold red.

Enzyme activity analysis of DkChi

In order to test the DkChi exo- and endo-chitinase activities, we performed the enzyme activity of DkChi using 4-MU-$(GlcNAC)_2$ and 4-MU-$(GlcNAC)_3$ substrates, respectively. As shown in pic. 5, both exo- and endo-chitinase activities increased with the DkChi protein concentration and reached 4.7 fold and 3.7 fold over their lowest levels, respectively. These data indicated that DkChi enzyme produced in *E. coli* was active and exhibited its native exo- and endo-chitinolytic activities.



Pic. 5**.** Enzymatic assay of DkChi purified from *E. coli* cells. Increasing amounts of the recombinant ChiA were incubated either with 4-MU-$(GlcNAc)_2$ to detect exo-chitinase activity (**a**) with 4-MU-$(GlcNAc)_3$ to detect endo-chitinase activity (**b**). Results are expressed as means±SE (n=3).

In vivo assays on larvae

The DkChi protein showed a strong insecticidal activity against *S. exigua*, *H. cunea*, *H. armigera* and *L. dispar*. The IC_{50} (50% lethal concentration) values of the larvae were 192.4, 305.3, 378.9 and 431.7ng/cm^2, respectively (Table 1).

Insect species	LC_{50} (ng/cm^2)[1]	Fl 95min-max[2]	Slope
Spodoptera exigua	192.4	68.3-587.7	2.5
Hyphantria cunea	305.3	98.6-578.8	2.3
Helicoverp armigera	378.9	74.1-543.2	3.4
Lymantria dispar	431.7	114.2-846.5	1.9

[1] LC_{50}, 50% lethal concentration
[2] Fl 95min-max, 95% confidence limit

Discussion

In this paper, DkChi (JN680874) from *Dendrolimus kikuchii* nucleopolyhedrovirus was characterized. Based on the alignment with HcNPV and other NPVs, several functional consensus motifs identified in baculovirus chitinase genes were found in the deduced DkChi full-length sequence. It contained an N-terminal secretion signal that was cleaved upon translation and a C-terminal ER-retention sequence (Pic. 1) that probably involved in retaining the enzyme inside the cell. ER-retention sequences have also been identified in the chitinases of *B. mori* NPV [27], AcNPV [14, 15] and HaSNPV [10]. Because of ER retention motif (KDEL), chitinase of AcMNPV was localized in the ER during infection, and deletion of KDEL motif resulted in earlier secretion into the media from infected cells [14, 15]. Furthermore, the two major domains: a PKD1 domain and a catalytic domain indicative of the family 18 glycohydrolase deduced on DkChi are likely to the known structure of SmchiA [28]. The PKD1 domain formed an immunoglobulin-like fold is involved in carbohydrate splitting and guiding the substrate into the catalytic groove [29, 30]. The catalytic domain formed a deep substrate-binding cleft [25, 26]. In addition, conserved tryptophan residues along the PKD1 fold and other aromatic residues in the catalytic domain were found on the surface of SmChiA and other chitinases [25, 26]. The conserved residues of SmChiA included Trp69 and Trp33 in the N-terminal domain and Trp245 in the catalytic domain playing vital role in the chitin binding and Phe-232 guiding the chitin chain into the catalytic cleft [25]. Young et al. reported ORF110 from *Epiphyas postvittana* nucleopolyhedrovirus (EppoNPV) had conserved residues Trp223, Trp236 and Tyr161 leading into the catalytic cleft and Trp158, Trp529, Trp266, Tyr410 and Trp388 forming the catalytic binding site [26]. Analogously, DkChi had the relative position of Trp-27 and Trp-60 along the immunoglobulin-like fold, Trp224, Trp237 and Tyr162 may also aid in feeding the insoluble chitin chain into catalytic pocket and thus to the active site and Trp159, Typ531, Trp277, Tyr411 and Trp389 probably forming hydrophobic interactions with hydrophobic faces of the alternating glucosamine units of chitin, thus producing the binding sites.

To obtain the biological activities, the truncated *DkChi* gene lacking the N-terminal signal peptide sequence and C-terminal ER-retention sequence (REEL) was expressed in *E. coli*. The recombinant DkChi protein stored as a small amount of inclusion bodies and large amount of soluble cytosolic components (Pic. 2). In order to avoid the multi-step renaturing processes to recover the enzyme activities, the recombinant protein was efficiently purified in its native form and further identified by SDS-PAGE and MALDI-TOF MS (Pic. 3 and Pic. 4). The DkChi protein showed both exo- and endo-chitinase activities using either 4-MU-$(GlcNAc)_2$ or 4-MU-$(GlcNAc)_3$ substrate, respectively (Pic. 5). It was consistent with the previous reports stating that baculovirus chitinases had both exo- and endo-chitinases activities [11, 12, 16].

The DkChi of DkNPV displayed an obvious insecticidal activity agianst *S*. *exigua*, *H*. *cunea*, *H*. *armigera* and *L*. *dispar* (Table 1). It indicated that DkChi had a huge potential in pest control. Previous studies had also showed the role of baculovirus chitinase. Rao et al. (2004) reported that ChiA of AcMNPV resulted in the decrease of larval body weight (LW) of *B*. *mori* at a dose of 0.56 μg/g LW and 100% mortality at a dose of 1μg/g LW [16]. ChiA from BmNPV could aid in degrading the chitinous PM lining the *B*. *mori* larval midgut [17]. Whether the insecticidal mechanism of DkChi is due to the damage of PM, it need to be further investigated.

In summary, these results presented in this work identified a new chitinase from DkNPV as candidate protein capable of protecting crops and forests against insect pests.

Acknowledgments

This work was supported by the Program of National Nature Science Foundation of China (grant no. 31200491) and State 863 Project funded by Ministry of Science and Technology (grant no. 2012AA101503) P.R. China.

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A NEW INSECT CELL LINE FROM PUPAL OVARY OF *SPODOPTERA EXIGUA* ESTABLISHED BY STIMULATION WITH *N*-METHYL-*N'*-NITRO-*N*-NITROSOGUANIDINE (MNNG)

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摘要：通过对甜菜夜蛾蛹卵巢原始细胞进行N-甲基-N'硝基-N-亚硝基胍（MNNG）处理建立了一种连续细胞系。蛹卵巢细胞用3.0μg/mlMNNG处理3天后形成单层细胞，然后开始培养去除MNNG的细胞60天，并连续培养30代。新建立的细胞系注册名为IOZCAS-Spex12，其包含有球形、梭形和卵圆形3种类型细胞。细胞群落倍增时间发生在71小时的对数生长阶段。PCR扩增DNA指纹图谱分析证明该细胞系初始细胞来自甜菜夜蛾。经感染研究该细胞系对甜菜夜蛾核型多角体病毒敏感。研究结果显示该细胞系对甜菜夜蛾和丫纹夜蛾核型多角体病毒高敏感，对斜纹夜蛾核型多角体病毒轻微敏感，对棉铃虫和美国白蛾核型多角体病毒不敏感。研究结果表明，应用MNNG处理技术可以克服目前连续增殖细胞的困难，并为稳定开发分离昆虫细胞提供了可能。

关键词：昆虫卵巢细胞系，核型多角体病毒，MNNG

НОВАЯ КЛЕТОЧНАЯ ЛИНИЯ ЗАЧАТКОВ ЯИЧНИКОВ У КУКОЛОК *SPODOPTERA EXIGUA* ПУТЕМ СТИМУЛЯЦИИ С ПОМОЩЬЮ *N*-МЕТИЛ-*N'*-НИТРО-*N*-НИТРОЗО ГУАДИНИНА

Непрерывную клеточную линию, полученную из яйцеводов куколки *Spodoptera exigua*, устанавливали путем обработки первичных клеток N-метил-N'-нитро-N-нитрозогуанидином (MNNG). Через три дня после обработки клеток 3,0 мкг / мл MNNG клетки образовывали монослой и первоначально были субкультивированы через 60 дней, после удаления MNNG с последующим субкультивированием в течение 30 повторностей. Установленная клеточная линия, обозначенная IOZCAS-Spex 12, состояла из смеси трех типов клеток, включая сферические, веретеновидные и овальные клетки. Было обнаружено, что время удвоения популяции линии клеток во время фазы его логарифмического роста составляет 71 час. Анализ цепной реакции ДНК-амплификации ДНК-амплификацией подтвердил, что новая клеточная линия возникла из *S. exigua*. Исследована восприимчивость клеток IOZCAS-Spex 12 к инфекции некоторыми нуклеополиэдро-вирусами. Результаты показали, что клеточная линия была очень восприимчива к заражению нуклеополиэдров вирусом *S.*

exigua и множественным нуклеолидоревирусом *Autographa californica*, слегка восприимчивым к заражению нуклеоположителем вируса *Spopoptera litura*, и не подвержена заражению нуклеополиэфировитами *Helicoverpa armigera* или *Hyphantria cunea* nucleopolyhedroviruses. Результаты этого исследования показывают, что лечение MNNG может преодолеть существующие ограничения на получение непрерывно пролиферирующих клеток и может открыть возможности для увековечения изолированных клеток насекомых.
Ключевые слова: клеточная линия клеток яичника, нуклеополиэдров, MNNG

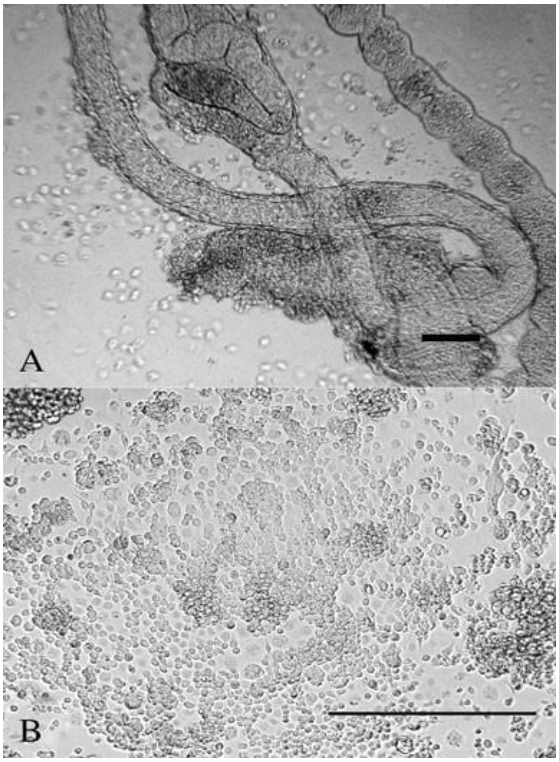
ABSTRACT: A continuous cell line derived from the pupal ovary of *Spodoptera exigua* was established by treating primary cells with N-methyl-N′-nitro-N- nitrosoguanidine (MNNG). Three days after treating cells with 3.0 μg/ml of MNNG, the cells formed a monolayer and were initially subcultured 60 d after the MNNG was removed, followed by subculturing for 30 passages. The established cell line, designated IOZCAS-Spex 12, consisted of a mixture of three types of cells, including spherical, spindle-shaped, and oval cells. The population doubling time of the cell line during its logarithmic growth phase was found to be 71 h. DNA amplification fingerprinting polymerase chain reaction analysis confirmed that the new cell line originated from S. exigua. Susceptibility of IOZCAS-Spex 12 cells to infection by certain nucleopolyhedroviruses was investigated. The results showed that the cell line was highly susceptible to infection by S. exigua nucleopolyhedrovirus and Autographa californica multiple nucleopolyhedrovirus, slightly susceptible to infection by Spodoptera litura nucleopolyhedrovirus, and not susceptible to infection by Helicoverpa armigera nucleopolyhedroviruses or Hyphantria cunea nucleopolyhedroviruses. The results of this study suggest that MNNG treatment may overcome existing limitations to obtaining continually proliferating cells and may open up the possibilities for immortalizing isolated insect cells.
Key words: Insect ovary cell line, Nucleopolyhedrovirus, MNNG

The use of insect cell lines is becoming more common in the fields of agriculture and biotechnology (Aucoin et al. 2010). Since the first insect cell line was established in the 1960 s, more than 500 insect cell lines have been developed from approximate 120 different insect species (Gaw et al. 1959; Grace 1962; Yeh et al. 2007). However, compared to mammalian cell lines, methods for establishing insect cell lines are still in their infancy (Simcox et al. 2008). Methods of in vitro chemical transformation in various mammalian cells are well established. For instance, N-methyl-N’-nitro-Nnitrosoguanidine (MNNG), a monofunctional alkylating agent that causes chromosomal DNA damage, has been used in in vitro cell culture systems to transform various types of normal or healthy cells into tumor cells (Gichner and Veleminsky 1982; Schar 2001). Carcinogenesis and the mechanisms of MNNG transformation have been extensively investigated through both in vitro and in vivo studies (Du et al. 1984; Su et al. 1995; Bunton and Wolfe 1996; Schar 2001; Izyumov and Talikina 2007).

However, few similar studies have examined the use of MNNG to establish insect cell lines. Although insect cells treated with carcinogens can survive for a certain time period, cell multiplication has not been observed, and attempts to establish a continuous insect cell line have failed (Mitsuhashi

2002). However, progress in gene technology has enabled the immortalization of cells by the introduction of oncogenes into other invertebrates. Tapay et al. (1995) obtained a continuous cell line from shrimp by transforming primary cultured lymphoid cells with simian virus (SV)-40 (T) antigen. It has been established that its development and progress are associated with the deregulation of many genes, as well as the mutation of oncogenes and loss of function of tumor suppressor genes (Early et al. 2008). However, the detailed molecular mechanism of establishing a cell line is not fully understood. Many important biological processes are involved in transformation and tumorigenesis, including cell cycle control, DNA damage repair, cell apoptosis, and signal transduction (Zhang et al. 2009a). The present study created a new insect cell line from primary cultured insect tissueusing MNNGtransformationand potentially describes a new method for establishing insect cell lines.

Successful cultures were initiated in June 2010. Initiation and maintenance of the cell lines were the same as previously described (Zhang et al. 2012). Cell migration occurred within 2 d of initiation of the cell culture. The migrated cells were either fibroblasts or hemocyte-like cells. They initially distributed themselves densely around tissue explants, gradually moved to the surrounding areas, and finally distributed over most of the flask after 30 d of culturing (Pic. 1A).



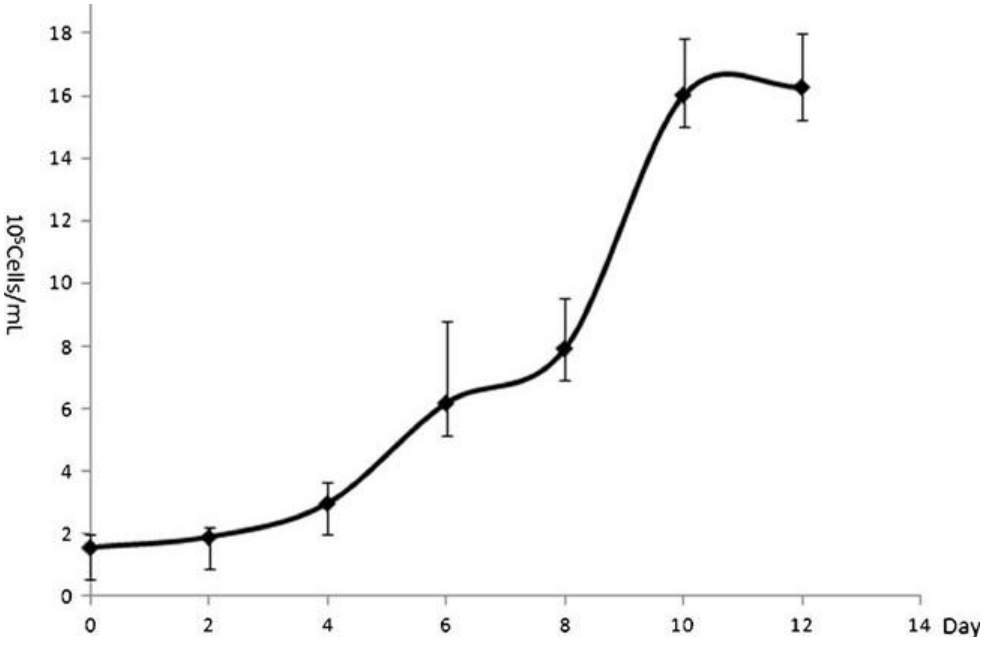
Pic. 1. **(A)** Cell migration that occurred within 2 d of cell culturing. The *scale bar* is 100 μm. (B) Cell monolayer of IOZCAS-Spex 12. The scale bar is 400 um.

When the cells approached confluence, they were treated with MNNG at a concentration of 1.0, 3.0, or 5.0 μg/ml for 3 d. After incubation with MNNG, the cells were thoroughly washed three times with fresh medium and cultured in growth medium at 27°C (Ming et al. 2006). The cultures were fed with one-half volume of fresh medium every 7 d. In the cultures treated with 3.0 μg/ml MNNG, most cells survived to day 30, although a few cells detached and degenerated. The surviving cells, most of which were polygonal or round, remained healthy, and some cells grew in volume by day 60. Dividing cells were continuously present, and cell survival time was significantly increased by the stimulation of MNNG. The first subculture was carried out 60 d after MNNG treatment and after the cells had reached confluence. The contents of the culture flask were then transferred to a new flask containing 2.0 ml fresh growth medium. The interval of time between the initial subculture to the tenth passage ranged from 10 to 15 d, depending on the growth rate of the cells. After the 11th passage, the cells proliferated more

rapidly, and thereafter, the interval of time between passages was 5 to 7 d using a ratio of cell suspension to fresh medium of 1:4 to 1:5 (Pic. 1B). The resulting cell line was designated IOZCAS-Spex 12.

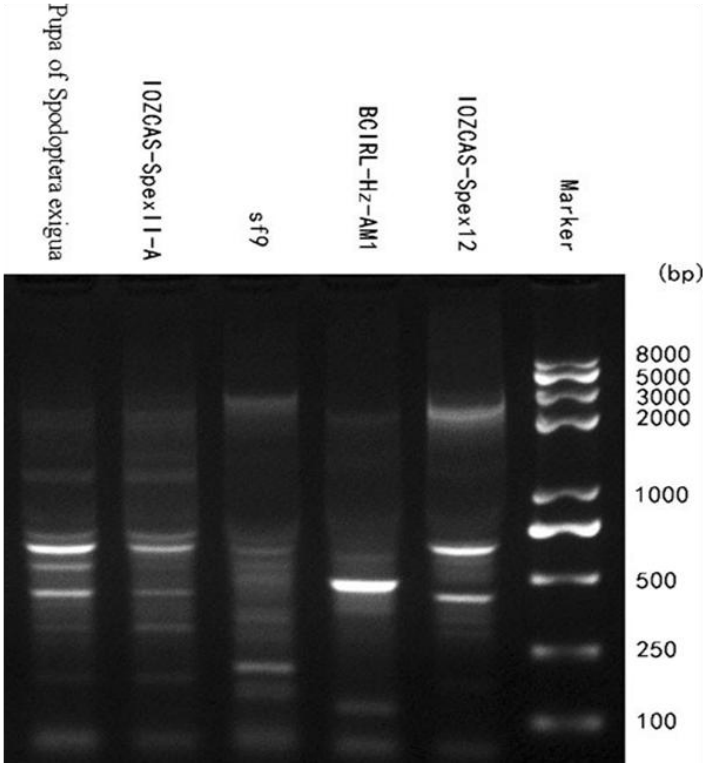
By acting directly on nucleic acids, MNNG is a strong mutagen that causes chromosomal DNA damage and can successfully induce in vitro cell transformation in several cell lines (Gichner and Veleminsky 1982; Du et al. 1984; Milo et al. 1992; Malik et al. 1997; Ming et al. 2006). Therefore, we used MNNG in our study to induce the division and transformation of cells cultured from the pupal ovary of Spodoptera exigua. In the presence of MNNG, the cultured cells grew well, and cells survived more than 30 passages. Both the concentration and timing of MNNG treatment were found to be important factors in determining the rates of proliferation and survival of the cultured cells. Cell division and the number of proliferating cells increased over time when the concentration of MNNG was 3.0 μg/ml. In the groups treated with 1.0 μg/ml MNNG, cells detached from the flasks by 30 d after MNNG treatment. Small rounded cells, but no dividing cells, were observed at day 60, and most cells in these cultures degenerated and eventually died at day 150. When the concentration of MNNG was 5.0 μg/ml or higher, most of the cells detached and degenerated by 3 d after treatment, showing that high concentrations of MNNG had a toxic effect on cells (Ming et al. 2006). In addition, the timing of stimulation seemed to be important. The most appropriate length of time for MNNG treatment in our study was 3 d after isolation. We found that MNNG treatment might overcome previously described limitations to obtaining continually proliferating insect cells and may open up the possibility of immortalizing isolated cells.

The morphology of the different cell types in the IOZCAS cultures was observed under phase contrast microscopy. The growth curve of the cell line was determined as previously described (Zhang et al. 2006a), and the cell population doubling time during the logarithmic growth phase was found to be 71 h (Pic. 2) (McIntosh and Ignoffo 1989). The cell line was found to have



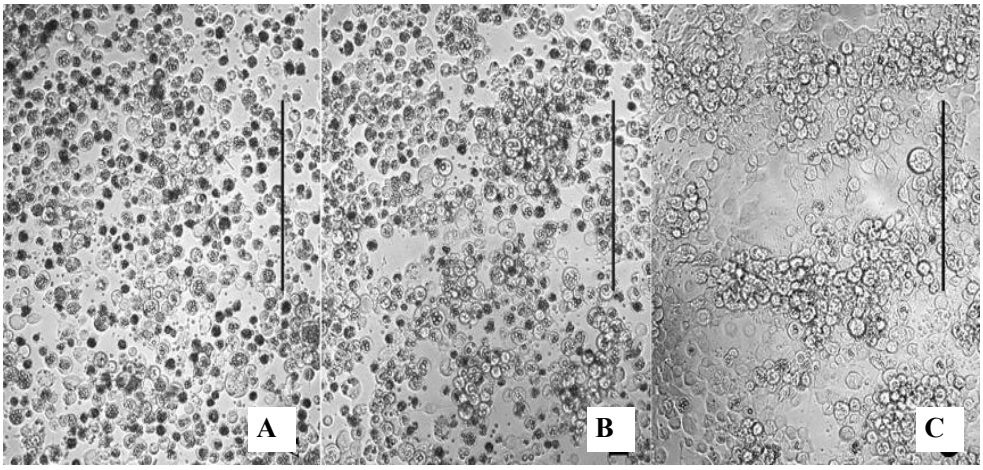
Pic. 2. The growth curves of the new cell line, IOZCAS-Spex 12.

originated from S. exigua by utilizing the DNA fingerprinting-PCR(DAF-PCR) method and comparing the band profiles of the cell line with its host, using the aldolase sequence as a primer (McIntosh et al. 1996; Liu et al. 2003). The PCR reaction conditions used in this study have been previously described (Zhang et al. 2006a). Three major bands at approximately 350, 450, and 700 bp of identity were shared (Pic. 3) between the new S. exigua cell line, another cell line from the same host (IOZCAS-Spex II-A), a cell clone from larval fat bodies of S. exigua Zhang et al. 2009b), and the homologous host (S. exigua pupa). A distinctive profile was seen compared to other cell lines maintained in the laboratory (Sf9, a cell line from Spodoptera frugiperda, Vaughn et al. 1977; BCIRL-Hz-AM1, a cell lines from Helicoverpa zea, McIntosh and Ignoffo 1983).



Pic. 3. DAF-PCR profiles of IOZCAS-Spex 12 cell lines and other cell lines in our laboratory, including Sf9, BCRIL-HzAM1, and IOZCAS-Spex II-A. The major bands identified are shared between the cell lines and their host, while the other cell lines show different profiles from IOZCAS-Spex 12 cells.

For determining the susceptibility of the cell line to nucleopolyhedroviruses, the viral inocula and inoculating process were as previously described (Zhang et al. 2006a, b). The viruses tested in this study include *S. exigua nucleopolyhedrovirus* (SeNPV), *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV), *Spodoptera litura nucleopolyhedrovirus* (SpltNPV), *Helicoverpa armigera nucleopolyhedrovirus* (HaNPV), and *Hyphantria cunea nucleopolyhedrovirus* (HcNPV). Typical cytopathogenic phenomena, such as enlarged nuclei with numerous occlusion bodies formed in the nuclei at the late stage of infection, were observed in the cultures inoculated with SeNPV, AcMNPV, and SpltNPV (Pic. 4 A–C). Susceptibility of the cell line to infection by these three viruses was further confirmed using a bioassay. The IOZCASSpex 12 cells were not susceptible to infection by HaNPV or HcNPV.



Pic. 4. The majority of cells are infected with SeNPV (A) AcMNPV (B), and SpltNPV (C). The scale bar is 200 μm.

Acknowledgments This research was jointly supported by the Young Scientists Fund of the National Natural Science Foundation of China (grant no. 30900947) and Beijing Municipal Natural Science Foundation (grant no. 5093028).

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Формат 70x108$^{1}/_{16}$ Объем 9.0 печ. л. Тираж 300 экз.

Отпечатано в ФБУ ВНИИЛМ
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