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Sensitivity of gypsy moth *Lymantria dispar* (L., 1758) larvae from geographically removed populations to nucleopolyhedrovirus (Lepidoptera: Erebidae, Lymantriinae)

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Abstract

Sensitivity of the gypsy moth (*Lymantria dispar*) larvae from geographical populations located in Khabarovsk and Novosibirsk, Russia, approximately 3800 kms apart, to its nucleopolyhedrovirus (LdNPV), was explored. Two isolates of LdNPV were used: "Altaiskiy" and "Novosibirskiy". The larvae were reared on natural diets from egg-masses that were collected during outbreaks in the above mentioned regions. Part of the gypsy moth eggs, from which the larvae were grown, was used for diagnostics of occult LdNPV by PCR method. It was shown that 48 ± 5% and 91 ± 7% of the embryos from Novosibirsk and Khabarovsk populations, respectively, were virus-positive. The LC_{50} value in II-instar larvae from Khabarovsk, infected with both isolates of LdNPV, was approximately 8 times lower than in the larvae population from Novosibirsk. High sensitivity to LdNPV may be connected to the high level of occult virus detected in insects from the Khabarovsk population.
KEY WORDS: Lepidoptera, Erebidae, Lymantriinae, *Lymantria dispar*, geographical populations, larvae, nucleopolyhedrovirus, sensitivity to LdNPV.

La sensibilidad al nucleopolihedrovirus de las larvas de la lagarta peluda *Lymantria dispar* (L., 1758) en poblaciones geográficamente separadas (Lepidoptera: Erebidae, Lymantriinae)

Resumen

Fue analizada la sensibilidad de las poblaciones geográficas de larvas de la lagarta peluda (*Lymantria dispar*) ubicadas en Khabarovsk y Novosibirsk, Rusia, separadas aproximadamente unos 3.800 kms, a sus nucleopolihedrovirus (LdNPV). Se usaron dos cepas de LdNPV: "Altaiskiy" y "Novosibirskiy". Las larvas fueron criadas con dietas naturales desde masas de huevos que fueron coleccionadas durante las eclosiones en las regiones mencionadas. Parte de los huevos de la lagarta peluda, de donde criamos las larvas, fue usada para el diagnóstico de LdNPV oculto por el método de PCR. Resultó que el 48 ± 5 % y 91 ± 7 % de los embriones de las poblaciones de Novosibirsk y de Khabarovsk, respectivamente, eran virus-positivos. El valor LC_{50} en larvas de II-estado de Khabarovsk, infectadas con ambas cepas de LdNPV, era aproximadamente 8 veces inferior al valor de la población de larvas de Novosibirsk. La alta sensibilidad para LdNPV podría estar relacionada con el alto nivel de virus oculto detectado en los insectos de la población de Khabarovsk.
PALABRAS CLAVE: Lepidoptera, Erebidae, Lymantriinae, *Lymantria dispar*, poblaciones geográficas, larva, nucleopolihedrovirus, sensibilidad al LdNPV.

Introduction

The gypsy moth is known as the most biologically and economically significant insect species. Outbreaks of this phyllophagous occurs seasonally in extensive areas. As a result, utter defoliation and mass withering of plantings can occur in areas spanning millions of hectares (MARSHALL, 1981). One of the leading factors influencing the population density of the gypsy moth is a disease caused by nucleopolyhedrovirus (LdNPV). Biopreparations for the biological control of gypsy moth population density have been created on the basis of LdNPV, and are currently under improvement (COOK *et al*., 2003).

The results of vertical transmission of viruses in some insect species under laboratory conditions were presented in review by KUKAN (1999). Nine out of 11 progenies from inoculated insects were infected with the virus, with the level of infection varying from 0.5% in *Pseudoplusia includens* to 57.1% in *Mythimra separata*. Vertical transmission of viruses was demonstrated in later works for *Trichoplusia ni* (FUXA *et al.*, 1999), *Bombyx mori* (KHURAD *et al.*, 2004) and other insects. Three papers on *Lymantria dispar* gave different results. SHAPIRO & ROBERTSON (1987) have shown that infection in II-instar larvae caused virus-induced mortality in the daughter generation from 4.7 to 11.5%. However, in a similar experiment by MURREY & ELKINTON (1989), transmission of virus to progeny was not shown (infection was < 2%). MYERS *et al.* (2000) reported that vertical transmission of overt infection occurred in 15% of egg masses produced by females inoculated with virus in larvae stage. The investigation of vertical virus transmission by PCR in *Plodia interpunctella* (BURDEN *et al.*, 2002) and *Spodoptera exigua* (CABODEVILLA *et al.*, 2011) has shown that virus was identified in progeny of infected insects over five generations (the period of observation).

PCR diagnostics for occult virus in populations of *Cydia pomonella* (British Columbia, Canada) (EASTWELLl *et al.*, 1999) and *Spodoptera litura* (Kagoshima Prefecture, Japan) (KOUASSI *et al*., 2009) has shown that in both cases 23% of insects have virus. PCR-screening of the natural *Mamestra brassicae* populations in England for occult virus has shown that viral DNA is present in insects from all 10 explored geographically removed regions (BURDEN *et al.*, 2003), but level of infection in populations varied from 50% to 100%.

For some moth species it was demonstrated that infection of caterpillars by a geterologous virus can activate its occult virus, which causes virus-induced mortality of the host (JURKOVIKOVA, 1979; COOPER *et al*., 2003; ILYINYKH & ULYANOVA, 2005; KOUASSI *et al*., 2009). This effect was demonstrated for *Adoxophyes orana* and *Barathra brassicae* (JURKOVIKOVA, 1979). The study of latent infection in *Malacosoma disstria* displayed activation of its occult virus when caterpillars were fed with virus from *Malacosoma californicum pluviale* (COOPER *et al*., 2003). Restriction analysis of viral DNA revealed that occult virus can be activated when *Lymantria monacha* larvae are infected with gypsy moth NPV (ILYINYKH & ULYANOVA, 2005). Finally, inoculation of laboratory stock of *Spodoptera litura* larvae with *Mythimna separata* NPV activated the occult virus (KOUASSI *et al*., 2009).

This work demonstrated that activation of occult virus by homologous NPV in infected caterpillars (carriers of occult virus) can therefore not be ruled out. Sensitivity of insects to NPV may depend on amount of insects with occult virus.

Materials and Methods

Insects

For determination of sensitivity to LdNPV, the insects from the Khabarovsk and Novosibirsk geographical populations of the gypsy moth, which are removed from each other by approximately 3800 kms, were used. In both cases the insect populations were at peak numbers. The collection of gypsy moth eggs was carried out during outbreaks in the territories of Novosibirsk Area and Khabarovsk Krai in the autumn (September) of 2006. The collected eggs of the gypsy moth were kept at +2C prior to commencing experiments (first 10 days of May). Preparation of eggs and cultivation of

larvae were performed as previously described (ILYINYKH *et al.*, 2004). During the experiments, the larvae of the Khabarovsk population were reared on branches of the Mongolian oak *Quercus mongolica*, and larvae of the Novosibirsk population were reared on branches of the birch *Betula pendula*. These plants represent one of the main food sources for the gypsy moth larvae in the Far East (the Khabarovsk population) and in Western Siberia (the Novosibirsk population). The attempt to rear Khabarovsk population larvae on branch of birch gave negative result (see results). The foliage of trees was washed with distilled water before use.

Larvae were reared in five-liter glass vessels, with 25 individuals in each vessel. The vessels were examined every day so as to eliminate the possibility of secondary infection. When necessary, the food was replaced and dead insects were removed.

The dead insects were examined by light microscopy (Biolam-R15; LOMO, Russia) using phase contrast to determine the cause of death. The experiments were conducted until the emergence of imagos. The quantitative details of the experiment are specified in Table 1.

Table 1.– Sensitivity of the gypsy moth larvae from Novosibirsk and Khabarovsk geographical populations to LdNPVs.

gypsy moth population name	LdNPVs isolate name	LdNPVs dosage (OBs/ml)	LdNPV-caused mortality (%)	LT_{50} value (± SE)	$lgLC_{50}$ value (99% CI)
Khabarovsk	Altaiskiy	2×10^8	99	9 ± 0.6	4.20 (3.61 – 4.83)
		2×10^7	97	11 ± 0.6	
		2×10^6	92	12.6 ± 0.8	
		2×10^5	77	18.4 ± 3.8	
		Control	0	–	
	Novosibirskiy	2×10^8	93	9.4 ± 0.5	5.77 (5.38 – 6.15)
		2×10^7	90	11.8 ± 2.1	
		2×10^6	82	13.6 ± 3.7	
		2×10^5	19	<	
		Control	0	–	
Novosibirsk	Altaiskiy	2×10^8	100	14.3 ± 2.6	5.80 (5.41 – 6.23)
		2×10^7	93	18 ± 2d	
		2×10^6	61	26.7 ± 5.1	
		2×10^5	37	<	
		Control	0	–	
	Novosibirskiy	2×10^8	94	17 ± 2.1	6.58 (6.32 – 6.94)
		2×10^7	53	26.3 ± 4.6	
		2×10^6	45	<	
		2×10^5	30	<	
		Control	0	–	

SE = Standard Error; CI = Confidence Interval

PREPARATION OF INSECTS FOR PCR

Sixty egg masses from the Novosibirsk and Khabarovsk populations of the gypsy moth were selected for analysis. A part of each of the gypsy moth egg masses, from which the insects were hatched for this work, was used for diagnostics of occult LdNPV by PCR. Each one was cleaned from fluff and was separated from each other. From separate egg-masses, 20 viable eggs were taken and surface sterilized with 0.25% NaOH on a magnetic mixer for 10 minutes. The eggs were then washed in sterile water and dried. Under sterile conditions the embryos were extracted from eggs and placed in 1.5 ml test tubes (Eppendorf), 20 individuals per tube. The samples were kept frozen at -70º Ñ before performing PCR.

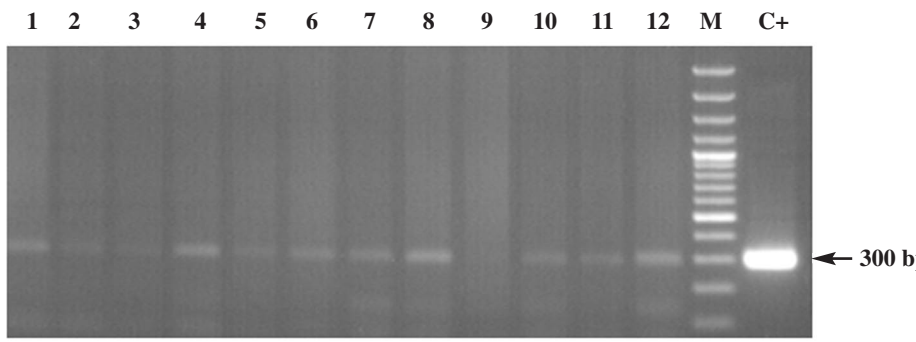

Figure 1.– Electrophoresis of PCR products amplified from the gene, encoding the LD 130 protein of Ld NPV from embryos of the gypsy moth (Khabarovsk population). Lanes 1-8, 10-12 – positive LdNVP tests; lane 9 – negative LdNVP test; lane M – marker O'GeneRuler 100bp Plus DNA Ladder (FERMENTAS, USA); lane C+ – positive control.

Total DNA from insect samples were extracted with a DNA isolation kit (Medigen Laboratory, Russia) according to the manufacturer's protocol. Amplification of a fragment of the envelope fusion protein, the Ld130 gene, was conducted in 20 l of buffer containing 10 l of PyroStart Fast PCR Master Mix (2X) (Fermentas, USA), 0.1 m of each primer and 27.5% of the total volume for the DNA. The design of the specific primers was performed based on the full genomic sequence of LdNPV deposited in GenBank (NC_001973). The primer sequences designed form the LdNPV genomic DNA for detection of the virus in biological samples are as follows: forward: 5'-CGGGCATCATCCGCGGCC-3' (127651–127668); reverse: 5'-CGCCCTCCAGCTCCGCGC-3' (127944–127927).

PCR was carried out in a DNA Engine thermal cycler (Bio-Rad, USA) for 1 cycle of 1 min at 94º C, then 37 PCR cycles at 94º C for 30 s, 68º C for 30 s, and 72º C for 30 s, with a final extension cycle at 72º C for 7 min. The size of amplified fragment was 294 base pairs.

LDNPV ISOLATES

In the given work two isolates of LdNPV were used: "Altaiskiy" and "Novosibirskiy". These viruses were obtained from the gypsy moth natural populations in 2004 during outbreaks in the territories of Gorniy Altai ("Altaiskiy" isolate) and Novosibirsk Area ("Novosibirskiy" isolate). LdNPV was extracted and purified from dead insects, using the sucrose gradient method described by KAWABARATA & MATSUMOTO (1973). The purified samples of LdNPV were stored at +2º C.

LdNPV aliquots were diluted with distilled water and used in experiment at four different concentrations: 2 x 10^5, 2 x 10^6, 2 x 10^7 and 2 x 10^8 of viral occlusion bodies per ml (OBs/ml). The amount of OBs is determined by light microscopy in a Goryaev's chamber (analogous to a Thoma chamber).

INFECTION OF LARVAE WITH LDNPV

Four standard branches of the gypsy moth larvae food plants were evenly portioned on a 0.25 m^2 polyethylene platform and treated with 2.5 ml of LdNPV suspension distributed with a 5 ml capacity manual sprayer. Infection was conducted two days following the molting of the larvae of the second stage. After drying, a branch of the plant was placed in individual container with gypsy moth larvae. The larvae were exposed to virus-contaminated foliage for 72 hours. Untreated insects were used as a control group. There were 125 individuals per virus concentration and control.

Statistical analysis

The following factors were used for estimation of the biological activity of LdNPV strains: LC_{50} and LT_{50}. LC_{50} is a concentration of LdNPV that kills half of the total number of insects after infection. LT_{50} is the time sufficient for death of half of the total number of insects after infection. The LC_{50} value was estimated by the Trimmed Spearman-Karber method (HAMILTON *et al.*, 1977) using SPEARMAN program (Montana State University, Bozeman, MT, USA). This method is a nonparametric statistical procedure that is effective for different concentration-response curve shapes (BEKETOV & LIESS, 2008). The threshold for significance used in this work was set at 0.01.

Results

The larvae of Khabarovsk population feeding on birch branch atå badly and all died at younger ages. NPV was diagnosed in 28 ± 3% of the dead insects.

Data on estimation of sensitivity of the larvae to LdNPVs are presented in Table 1. The total death rate of insects from LdNPV infection in these experiments varied from 19% to 100%, in accordance with concentration of the virus which was used for the larvae treatment. Mortality of insects in uninfected control groups was not due to LdNPV.

Infection of individuals from the Khabarovsk and Novosibirsk populations with high virus concentrations (2 x 10^8, 2 x 10^7 Obs/ml) resulted in LT_{50} values from 9 ± 0.6 to 11.8 ± 2.1 days and from 14.3 ± 2.6 to 18 ± 2 days accordingly. This value was 12.6 ± 0.8 – 18.4 ± 3.8 days after infection of the larvae of the Khabarovsk population with the LdNPV "Altaiskiy" strain at a lower concentration. In the majority of other cases, death rate of insects did not reach 50%.

The LC_{50} determination in experimental infection of larvae with the "Altaiskiy" strain revealed that this factor for Khabarovsk population was approximately 15 times lower than for the Novosibirsk population (4.20 (3.61-4.83) and 5.80 (5.41-6.23), accordingly).

The LC_{50} value of insects infected with the "Novosibirskiy" strain was approximately 8 times lower for the Khabarovsk population in comparison to the caterpillars of the Novosibirsk population (5.77 (5.38-6.15) and 6.58 (6.32-6.94), respectively). Thereby, conducted experiments have shown that the gypsy moth larvae of Khabarovsk geographical population are more sensitive to LdNPV, than insects of the Novosibirsk population.

The study of the occult virus by PCR method (Fig. 1) has revealed that the amount of virus-positive individuals among insects of the Novosibirsk and Khabarovsk populations was 48 ± 5% and 91 ± 7%, respectively.

Discussion

It was shown that larval sensitivity to NPVs can vary with species of food plants. For instance, KEATING *et al.* (1990) demonstrated that the mortality of gypsy moth caterpillars feeding on leaves of red maple *Acer rubrum*, red oak *Quercus rubra*, and black oak *Q. velutina* was twice lower compared to those feeding on leaves of quaking aspen *Populus tremuloides*. High level of pH and phenols (particularly, hydrolyzable tannins) in the leaves were proposed as the factors inhibiting viral infection in the caterpillars (KEATING *et al.*, 1990). Other examples also demonstrate that insect mortality can vary when viruses are ingested on different host plants (CORY & HOOVER, 2006).

We have not found in the literature facts on the composition of hydrolyzable tannins in leaves of *Quercus mongolica* and *Betula pendula*. For the other species of birch it is known that contents of these components in the leaves of mountain birch *Betula pubescens* are comparatively high (SALMINEN *et al.*, 2001). Therefore, based on available literature data, a priori it is possible to expect equally high levels of hydrolyzable tannins in leaves of *Quercus mongolica* and *Betula pendula*. However, it cannot be excluded that some differences in the biochemical composition of the leaves of these plants may be a factor responsible for the variation of sensitivity of the gypsy moth larvae to LdNPV.

In natural populations of the gypsy moth from the territories of the Far East and Western Siberia, the role of LdNPV in insect population density is substantially different. In the Far Eastern populations, LdNPV is one of the key factors affecting population density dynamics (ILYINYKH *et al.*, 2011), while in the Western Siberian populations, the mortality of insects from LdNPV is not significant (ILYINYKH *et al*., 2004). The probabilities of infection of these insect populations by NPVs differ, which may be attributed to genetically-based variations. It was shown in laboratory experiments for some species of insects and their baculoviruses that sensitivity to NPVs can decrease after infection of several consequent generations of insects with these viruses (MILKS & MYERS, 2001).

As mentioned above, the main food plant for gypsy moth larvae in the Far East is *Quercus mongolica*. So NPV-caused mortality of far eastern population larvae reared on leaves of birch, was probably the result of activation of occult infection. For the gypsy moth, it is known that rearing larvae in laboratory on a nonspecific diet can activate occult viral infection (LINDROTH *et al*., 1991; ILYINYKH *et al*., 2004).

At this stage we can only speculate as to the reasons for differences in insect sensitivity to NPV. However, the higher sensitivity of insects from Khabarovsk population to LdNPV can be related to a larger number of virus-carrying individuals in comparison with the Novosibirsk population. Presumably, there is a synergic effect that occurs whereby infection of larvae with LdNPV causes the activation of occult virus. Furthermore, this effect reveals itself to a greater degree among insects with a higher prevalence of occult virus.

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