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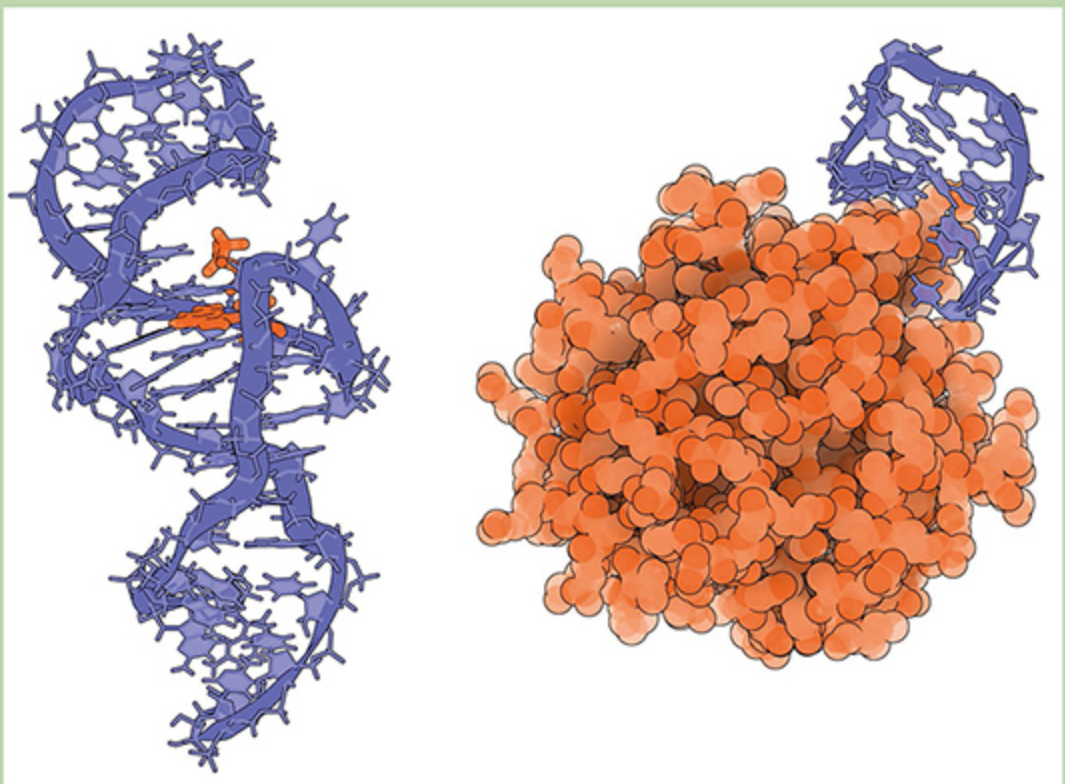

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Full-text article

MOLECULAR DETECTION OF ENDOSYMBIONTS IN LOCAL POPULATIONS OF *HELICOVERPA ARMIGERA* (LEPIDOPTERA: NOCTUIDAE) IN EUROPEAN PART OF RUSSIA

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Cotton bollworm *Helicoverpa armigera* is one of the most polyphagous and cosmopolitan pests. Intracellular endosymbionts are widespread in Lepidoptera, often playing an important role in their dynamics. The prevalence of endosymbionts of cotton bollworm in Russia was not investigated. Cotton bollworm larvae and adults were collected in 2018–2020 in Krasnodar Area, and in Voronezh and Saratov Regions (from 131 to 170 insects) and analyzed by PCR using sets of group-specific primers for baculoviruses (locus *lef8*), bacteria of the genus of *Wolbachia* (locus *wsp*), and microsporidia (locus SSU rRNA). Level of infection with baculoviruses was 16% for the sample of 32 individuals collected in Temryuk District of Krasnodar Area in 2018. The infection rate of the entire sample of 170 individuals was 2.9%. The *lef8* locus demonstrated 98.7–99.6% of sequence similarity to the nuclear polyhedrosis virus isolates from the cotton bollworm and American bollworm. Among the tested 131 insects, bacteria of the genus of *Wolbachia* were not detected. PCR screening for microsporidia revealed one positive larvae among 19 insects collected in Krasnoarmeysk District of Krasnodar Area in 2019, which corresponded to the prevalence of 5%. Partial sequencing of the genes coding for SSU rRNA and largest subunit RNA polymerase II made it possible to identify the new isolate as *N. bombycis*.

Keywords: obligate intracellular parasites, entomopathogenic microorganisms, pests lepidoptera, natural infection, nuclear polyhedrosis virus, Microsporidia, *Wolbachia*, *Nosema bombycis*

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Introduction

Cotton bollworm *Helicoverpa armigera* (Noctuoidea: Noctuidae) is among the most polyphagous and cosmopolitan pests (Cunningham, Zalucki 2014; Gomes et al., 2017). This is a multivoltine species characterized by high ecological plasticity, which allows the insect to adapt easily to changing environmental conditions and reach a high abundance (Chenkin et al., 1990; Farrow, Daly, 1987; Jones et al., 2018). The cotton bollworm is one of the most dangerous agricultural pests in Russia and other countries of Europe, Asia, Africa, Australia etc. (Fitt, 1989; Tay et al., 2013; Arnemann et al., 2016; Murúa et al., 2014; Czepak et al., 2013). According to different sources, the number of plant species damaged by this pest ranges between 120 and 180 species (Singh et al., 2002; Wu et al., 2008; Murúa et al., 2014). The most preferred crops are cotton, tomatoes, cereals, such as corn and sorghum, as well as soybean, chickpea and other legumes (Riaz et al., 2021). The annual global cost of controlling this pest, together with crop losses, is estimated at US$5 billion (Murúa et al., 2014; Haile et al., 2021). Intensive and sometimes unreasonable use of broad-spectrum synthetic pesticides reduces the effectiveness of natural enemies and biocontrol agents, while the target pest species develop resistance to a wide range of insecticides (Armes et al., 1996; Ahmed et al., 2004; Yang et al., 2013). Solving these problems requires improvement of the synthetic pesticides range and their rational use to preserve the natural enemies of *H. armigera* in different agroecosystems (Mohan et al., 2008; Williams et al., 2022), as well as development of alternative, environmentally friendly approaches to population management (Binod et al., 2007; Yu et al., 2008; Patil, Jadhav, 2015; Suryanarayanan et al., 2016; Knox et al., 2016; Kolosov et al., 2017; Agasieva et al., 2019). To understand the main patterns of population dynamics, improve forecasting systems and identify new forms of potential sources of microbiological formulations, it is necessary to perform screening of the pest populations aimed at identification of pathogenic microorganisms of the main groups.

Among the obligate intracellular symbionts, which are the most widespread and frequently found in insects, three groups deserve attention in the first place.

The first group includes the nuclear polyhedrosis (NPV) and granulosis viruses from the Baculoviridae family of double-stranded DNA viruses that infect insects from the orders of Lepidoptera, Hymenoptera, and Diptera (Van Regenmortel et al., 2000). They serve as the basis of microbial formulations against Lepidoptera pests, including the cotton bollworm, which are widely used worldwide (Chen et al., 2000; Shapiro et al., 2002; Yu et al., 2015; Kolosov et al., 2017; Eroğlu et al., 2019). NPV populations can grow rapidly, increasing it number at billion-fold rate per insect. Up to three such viral "generations" can be multiplied in one generation of insects (Harper, 1987), which provides in vivo large-scale propagation of baculoviruses and makes them the promising agents for biological plant protection (Eroglu et al., 2018). Despite the isolation of numerous NPV strains from the cotton bollworm in various parts of the world and studies of their genetic polymorphism and effectiveness in terms of combating this pest (Leslie Hayes, Bell, 1994; Moscardi, 1999; Erlandson, 2009; Baillie, Bouwer, 2012, Arrizubieta et al., 2014; Ardisson-Araújo et al., 2015), assessment of natural prevalence levels is usually not carried out. Specific data on the levels of natural

infection of cotton bollworm populations are missing. For other members of the Noctuidae family, there are data on the prevalence of nuclear polyhedrosis virus for *Spodoptera frugiperda* in Louisiana, where the virus prevalence ranged from 50 to 68%, being higher than that of the other pathogens (Fuxa, 1982). In other works, the level of occurrence of NPV in lepidopteran was estimated after artificial introductions of viral particles, which did not allow estimating the natural prevalence rates (Fuxa and Richter, 1999; Cherry et al, 2000).

The second group, bacteria of the genus *Wolbachia*, belong to widespread endosymbionts of arthropods (Bouchon et al., 1998), and infect according to various estimates, from 40 to 65% of the arthropod species (Hilgenboecker et al., 2008; Werren et al., 2008; Zug, Hammerstein 2012). The effects of *Wolbachia* on insects including Lepidoptera, are very diverse (Hiroki et al., 2004; Charlat et al., 2006, 2007; Narita et al., 2007; Graham, Wilson, 2012; Salunkhe et al., 2014; Arai et al., 2019), and the study of these bacteria is of interest both from theoretical and practical points of view. The prevalence of *Wolbachia* in lepidopteran populations varies from almost complete absence to 100% infection (Tagami, Miura, 2004; Salunke et al., 2012; Ahmed et al., 2015; Solovyev et al., 2015; Ilinsky, Kosterin, 2017; Tokarev et al., 2017; Bykov et al., 2020). For example, in *Dendrolimus superans*, a high level of infection with *Wolbachia* (69–100%) has been shown to be maintained in geographically distant populations for several years (Bykov et al., 2020). For *Aporia crataegi*, the frequency of *Wolbachia* occurrence was very low: out of 376 samples collected in 10 regions of Russia, only eight *Wolbachia*-positive insects were found in Yakutia, Buryatia, Sverdlovsk and Kaliningrad Regions (Bykov et al., 2021). In *Loxostege sticticalis*, the prevalence of *Wolbachia* varied from 21 to 40% in Asian and from 0 to 47% in European parts of Russia (Malysh et al., 2020). Analysis of the sample of 257 individuals for the presence of *Wolbachia* in *Hypolimnas bolina* females collected from the wild habitat showed that 99% of the females were infected (Dyson, Hurst, 2004). The presence of endosymbiotic bacteria of the genus of *Wolbachia* was also found in populations of stem borers of the genus *Ostrinia.* In various geographic populations, endosymbiont prevalence ranged from 2.9 (N=34) to 65.8% (N=38), with three of the four habitats showing a significantly higher level of infection for *O. scapulalis* as compared to *O. nubilalis* (Tokarev et al., 2017). *Pieris rapae* in Japan was infected with *Wolbachia* with the prevalence of 0–3% (Tagami, Miura, 2004). In addition, in the Japanese populations of the gypsy moth (*Lymantria dispar japonica* and *L. postalba*), the presence of *Wolbachia* was not revealed (Ilinsky et al, 2017).

The third group is the microsporidia, parasitic protists related to fungi. They parasitize the representatives of all major taxa of Animalia kingdom, including higher vertebrates and humans (Issi, 2020). The largest number of microsporidia was found in arthropods (Wittner, 1999), and many species are highly pathogenic to these hosts and significantly affect their populations. Interest in the study of microsporidia has notably increased recently due to the understanding of their role as dangerous pathogens of humans and economically significant species of vertebrates and invertebrates. They are also widely exploited as a model of intracellular parasites demonstrating the maximum level of genome and cell minimization (Wittner, 1999; Becnel, Andreadis, 2014). The role of infection with microsporidia in the host density dynamics has been studied well for several lepidopterans (Issi, 1986; Frolov et al., 2008; Lipa, Madziara-Borusiewicz, 1976; Zelinskaya, 1980; Solter et al., 1997; 2010; Van Frankenhuyzen et al., 2007; Kermani et al., 2013; Simoes et al., 2015; Hopper et al., 2016; Malysh et al., 2021). In particular, in the stem borers of the genus of *Ostrinia*, the levels of microsporidia infection in Russia ranged from 3.0 to 17.2% in 2005–2010 (Malysh et al., 2011) and from 0 to 16% in 2011–2016 (Grushevaya et al., 2018). PCR analysis of 98 individuals of *L. sticticalis* for the presence of microsporidia was positive for 7% of the samples (Malysh et al., 2019). The prevalence of microsporidiosis in *Bombyx mori* in India was about 15–20% (Bhat et al., 2009). In *Archips xylosteana* in Bulgaria, the prevalence of microsporidiosis was 3% for the sample of 791 individuals (Pilarska, 2017). In Japanese populations of *Lymantria* spp., microsporidia infection was not detected (Ilinsky et al, 2017), although they are known in European and North American populations (McManus, Solter, 2003). In the susceptibility bioassays of the cotton bollworm, isolates of microsporidia from different hosts were exploited. However, when spotting microsporidia infections of the cotton bollworm under natural conditions, the frequency data were not indicated (Issi, Nilova, 1967; Gaugler, Brooks, 1975; Lee, Anstee, 1992; Mitchell, Cali, 1994; Rabindra, Jayaraj, 1994; Pei et al., 2021).

Studies of natural infections by viruses and microorganisms in populations of this pest in the Former Soviet Union Countries in the 21st century are restricted to the detection of new isolates of baculoviruses, most of which were done using laboratory-maintained insect cultures of Central Asian origin (Kolosov et al., 2017). The aim of this work was to assess the natural occurrence of baculoviruses, *Wolbachia* spp. and microsporidia in local populations of the cotton bollworm in the European part of Russia using molecular markers.

Materials and methods

To detect the presence of entomopathogens in cotton bollworm populations, *H. armigera* larvae were collected in maize stands in five localities of Krasnodar Area, Voronezh and Saratov Regions, and adults were caught on the pheromone trap at one point, Gulkevichi Region of Krasnodar Area in 2019 (Fig. 1). The total amount of collected material was 170 individuals. Insects were stored either dry at room temperature without preservatives or in 90% ethanol. Total DNA was extracted using a simplified protocol of Sambrook et al. (1989) without addition of phenol, with adjustments in the volumes of DNA washing agents (Malysh et al., 2019). Samples were homogenized in 100 µl of CTAB (Cetyl Trimethyl Ammonium Bromide). Then, 500 µl of CTAB + β-mercaptoethanol (final concentration 0.2%) were added and incubated at a +65 °C for 2 hours, consequently washed with a mixture of chloroform and isoamyl alcohol (24:1), precipitated with ethanol and resuspended in 50 µl of ultra-purified water. The DNA solution was used for PCR analysis. The PCR mix consisted of 4 µl of DNA, 5 µl of DreamTaq Green PCR Master Mix, and 0.5 µl of primers (forward and reverse). For the analysis, only half of each individual sample was used, saving the other half for further analysis in case the microsporidia spores are detected.

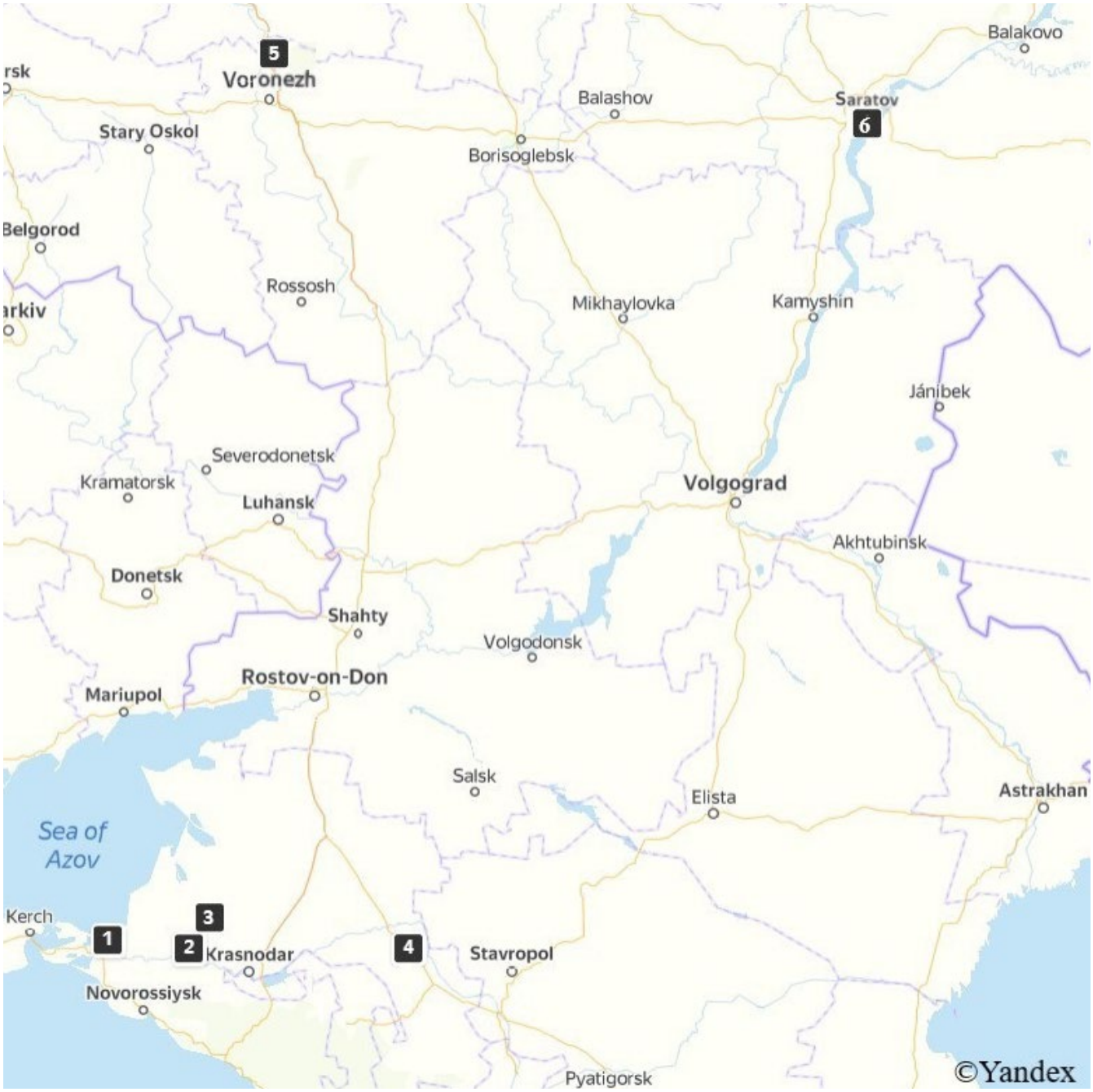

Figure 1. Sampling sites of *Helicoverpa armigera* larvae and adults in Temryuk (1), Slavyansk (2), Krasnoarmeysk (3), and Gulkevichi Districts (4) of Krasnodar Area, Ramon District of Voronezh Region (5), Engels District of Saratov Region (6)

Рисунок 1. Места отборов проб гусениц и имаго *Helicoverpa armigera* в Темрюкском (1), Славянском (2), Красноармейском (3) и Гулькевичском (4) районах Краснодарского края, Рамонском районе Воронежской области (5) и Энгельском районе Саратовской области (6)

The DNA quality of individual samples was checked by PCR with LepF1:LepR1 primers flanking the Metazoa mitochondrial DNA fragment (Hebert et al., 2004). Testing for the presence of *Wolbachia*, as well as baculoviral and microsporidian infections, was carried out by PCR amplification with the following primer sets (Table 1):

- to determine the presence of baculoviruses, primers L8F2:L8R2 were used, flanking the conservative region of the late elongation factor gene (*lef8*). As a reference (positive control), we used DNA samples of the cotton bollworm nuclear polyhedrosis virus, isolate HS-18 (kindly provided by Kolosov A.V., FBSI SRC VB "Vector" of Rospotrebnadzor);

- to determine the presence of microsporidia, standard primers 18f:1047r were used, to amplify part of the small subunit rRNA gene (SSU rRNA). The positive control was the DNA sample of microsporidia *Nosema pyrausta* from the corn borer (kindly provided by I.V. Grushevaya, All-Russian Institute of Plant Protection). For more accurate identification, primers nvRPb f1 were used: nvRPB r1 to the gene fragment of the large subunit of RNA polymerase II (*rpb1*);

- the analyses for the presence of *Wolbachia*, were carried out by amplification with the wsp81F:wsp691 primer set specific for the *Wolbachia* surface protein (*wsp*) locus. *Wolbachia* DNA samples from the corn borer (kindly provided by I.V. Grushevaya) were used as a positive control.

Table 1. Primers used for detection of endosymbionts of cotton bollworm *Helicoverpa armigera*

Таблица 1. Праймеры, используемые для диагностики эндосимбионтов хлопковой совки *Helicoverpa armigera*

Primer name Название праймера	5'-3' Primer sequence 5'-3'последовательность праймера	Target, amplicon size Цель, размер ампликона	Reference Ссылка
L8F2	GTAAAACGACGGCCAGTNNNACNRCNGARGAYCC	*Baculovirus* late elongation factor, ~500 bp	Herniou et al., 2004
L8R2	AACAGCTATGACCATGMMNCCYTTYTGNCCRTG		Herniou et al., 2004
wsp 81F	TGGTCCAATAAGTGATGAAGAAAC	*Wolbachia* surface protein, ~600 bp	Zhou et al., 1998
wsp 691R	AAAAATTAAACGCTACTCCA		Zhou et al., 1998
18f	GTTGATTCTGCCTGACG	Microsporidia small subunit rRNA, ~900 bp	Weiss, Vossbrinck, 1999
1047r	AACGGCCATGCACAC		Weiss, Vossbrinck, 1999
nvRPB1F1	CCWATGTTYCATGTYGGTTA'	RNA polymerase II largest subunit, ~700 bp	Tokarev et al., 2019
nvRPB1R1	TAATTACAGACCTGGCACT		

The amplification program was the same for all primers: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, elongation at 72 °C for 1 min, and final elongation step of 72 °C for 5 min.

The amplicons were visualized using electrophoresis in 1 % agarose gels with GeneRuler Ladder Mix molecular weight marker, 75–20000 bp (Thermo Fisher Scientific). Amplicons in the gel of about 500 bp (primers L8F2:L8R2), 600 bp (wsp81F:wsp691), 900 bp (18f:1047r) and 700 bp (nvRPB1F1:nvRPB1F1) were excised with a scalpel and frozen until further purification. The cut sections of the gel were melted in a 3 M solution of guanidine isothiocyanate, and the amplicons were purified by the silica sorption method (Vogelstein, Gillepsie, 1979). The purified amplicons were sequenced at the Core Centrum «Genomic Technologies, Proteomics and Cell Biology» of the All-Russian Institute of Agricultural Microbiology in both directions by a standard method of chain termination (Sanger et al., 1977) using an ABI Prism 3500 genetic analyzer. The obtained sequencing chromatograms were analyzed using the BioEdit software (Hall, 1999). The search for homologous sequences in GenBank was performed on the NCBI server using the built-in BLAST utility using the megablast and blast algorithms (Altschul et al., 1990).

To compare the morphometric characteristics of microsporidian spores, the length and width of at least 10 spores of the new isolate from the cotton bollworm were measured, and compared to the *N. bombycis* spores from the silkworm culture at the Research Institute of Sericulture (Tashkent, Uzbekistan), kindly provided by I.V. Senderskiy (All-Russian Institute of Plant Protection).

Results

The quality of DNA samples was confirmed by amplification of the DNA fragment of insects with primers LepF1:LepR1. In the most of samples, no positive signals or nonspecific reactions with non-target DNA were observed while diagnosing endosymbionts with corresponding primers. However, a few sequences of amplicons of the expected size amplified with baculovirus- and *Wolbachia*-specific primers, matched DNA fragments of the host insect or intestinal bacteria and were excluded from the study.

In particular, a number of amplicons positive reaction for baculoviruses was registered in 5 out of 32 samples from one sample of Temryuk District of Krasnodar Area in 2018. This corresponds to 16 % prevalence, and to 2.9 % if to consider the entire sample of 170 tested insects (Table 2).

Amplified fragments of the *lef8* gene were sequenced. The obtained sequences demonstrated high levels of identity with homologous regions of genomes of numerous viral isolates designated in GenBank as cotton bollworm nuclear polyhedrosis viruses (NPVs) (*Helicoverpa armigera* nucleopolyhedrovirus, HearNPV) or the American cotton bollworm NPVs (*Helicoverpa zea* nucleopolyhedrovirus, HzNPV). Both isolates derive from various representatives of closely related species of the *Heliothis/Helicoverpa* complex, and from *Hyblea puera* (Hyblaeoidea: Hyblaeidae). Alignment of 380 nucleotides showed 100 % identity of HS-18 strain used as standard in this study (1) with the corresponding fragment of the whole genome sequence deposited earlier for this strain in GenBank (accession # KJ004000) and (2) with some other HzNPV isolates (# KM596835) and (4) HearNPV (# KU738904 and # KJ922128). Isolates from Temryuk identified in this work contained two A/G transitions, one of which was not found in other isolates (Table 3). The similarity of the Temryuk isolates to the HzNPV and HearNPV sequences from GenBank, was 98.7–99.6 % (Table 4). To understand the genetic differentiation of HzNPV and HearNPV at the genome level, a BLAST analysis of the whole genome sequence of HS-18 was additionally performed, which showed a similarity of >99.9 % with HzNPV and >99.6 % with HearNPV (Table 5).

For assessing the prevalence of bacteria of the genus of *Wolbachia*, 131 individual DNA samples were tested. All of them produced a positive reaction with primers LepF1: LepR1 demonstrating thus suitability of the samples for PCR amplification. None of these samples produced a specific signal with *Wolbachia*-specific primers that could be confirmed by sequencing. At the same time, a sample of genomic DNA of a *Wolbachia*-infected corn borer, used as a positive control, gave a signal of the expected size in all the experiments performed. Thus we consider the negative result of *Wolbachia* detection in bollworm samples as reliable.

PCR screening for microsporidia revealed one positive signal for the sample from Krasnoarmeysk District of Krasnodar Area, obtained in 2019. Prevalence level in this sample equaled to 5.2 % (N=19), and for the whole dataset of 168 individuals – to 0.6 %. Sequencing the SSU rRNA gene fragment showed 100 % identity to the microsporidium *N. bombycis* from the silkworm *B. mori*, as well as to

Table 2. Size of analyzed samples from local populations of the cotton bollworm

Sampling site	Year	Stage	Number of analyzed samples* (N)		
			baculoviruses	microsporidia	*Wolbachia*
Krasnodar Area, Temryuk District	2018	larvae	32(5)	32	32
Krasnodar Area, Slavyansk District	2018	larvae	30	30	-
	2020	larvae	9	-	-
Krasnodar Area, Gulkevichi District	2019	adults (from traps)	30	30	30
Krasnodar Area, Krasnoarmeysk District	2019	larvae	12	19(1)	12
Voronezh Region, Ramon District	2019	larvae	29	29	29
Saratov Region, Engels District	2020	larvae	28	28	28
TOTAL			170(5)	168(1)	131(0)

* in brackets is the number of verified positive samples (if any).

Таблица 2. Объем проанализированных выборок локальных популяций хлопковой совки

Место сбора	Год сбора	Стадия развития	Объем выборки* при анализе на		
			бакуловирусы	микроспоридий	вольбахию
Краснодарский край, Темрюкский район	2018	Гусеницы	32(5)	32	32
Краснодарский край, Славянский район	2018	Гусеницы	30	30	-
	2020	Гусеницы	9	-	-
Краснодарский край, Гулькевичский район	2019	Имаго (из ловушек)	30	30	30
Краснодарский край, Красноармейский район	2019	Гусеницы	12	19(1)	12
Воронежская область, Рамонский район	2019	Гусеницы	29	29	29
Саратовская область, Энгельский район	2020	Гусеницы	28	28	28
ИТОГО			170(5)	168(1)	131(0)

* в скобках указано количество верифицированных положительных проб (при наличии).

Table 3. Polymorphism of the nucleotide sequences of the *lef8* gene fragment of the *Helicoverpa armigera* nucleopolyhedrovirus isolates obtained in the present study from Krasnodar Area (TEMRYUK21…32) and standard strain HS-18 (VECTOR), as well as those accessible through GenBank (annotated with accession number and host species)

Таблица 3. Полиморфизм нуклеотидных последовательностей фрагмента гена *lef8* вируса ядерного полиэдроза хлопковой совки, полученных в настоящей работе для изолятов из Краснодарского края (TEMRYUK21…32) и эталонного штамма ХС-18 (VECTOR), а также доступных в GenBank (указан номер доступа и вид насекомого-хозяина)

GenBank Accession # or strain name Номер доступа в GenBank или название изолята	Host species Вид хозяина	Nucleotide position as in reference sequence KJ004000* Положение нуклеотида относительно референсного сиквенса KJ004000*								
		32305	32308	32387	32389	32419	32488	32509	32524	32602
KJ004000 (HS-18)	*Helicoverpa zea*	C	G	T	A	G	T	G	C	G
VECTOR (HS-18 in this study)	*Helicoverpa armigera*	C	G	T	A	G	T	G	C	G
TEMRYUK21	*Helicoverpa armigera*	C	**A**	T	**G**	G	T	G	C	G
TEMRYUK24	*Helicoverpa armigera*	C	**A**	T	**G**	G	T	G	C	G
TEMRYUK32	*Helicoverpa armigera*	C	**A**	T	**G**	G	T	G	C	G
KU738904	*Helicoverpa*	C	G	T	A	G	T	G	C	G
KM596835	*Helicoverpa zea*	C	G	T	A	G	T	G	C	G
KJ922128	*Helicoverpa armigera*	C	G	T	A	G	T	G	C	G
KM357512	*Helicoverpa armigera*	C	G	T	**G**	G	T	G	C	G
AY118080	*Helicoverpa armigera*	C	G	T	**G**	G	T	**A**	C	G
KT013224	*Helicoverpa armigera*	C	G	T	**G**	**A**	T	G	C	G
KJ701031	*Helicoverpa armigera*	C	G	T	**G**	G	T	G	C	G
MK507817	*Helicoverpa armigera*	**T**	G	T	**G**	G	T	G	**T**	**A**
MG569706	*Helicoverpa assulta*	C	G	**A**	**G**	G	**C**	**A**	**T**	G
MT810812	*Helicoverpa armigera*	C	G	**A**	**G**	G	**C**	**A**	C	G
MH254887	*Hyblaea puera*	C	G	T	**G**	**A**	T	G	C	G

*The unique polymorphic position of the newly found baculovirus variants is highlighted with gray background.
*Уникальная полиморфная позиция вновь найденных вариантов бакуловируса отмечена серым фоном.

Table 4. The nucleotide sequences of the l*ef8* gene of isolates of the *Helicoverpa armigera* nuclear polyhedrosis virus available in GenBank, used for comparative analysis in this work

Species, isolate	Host	Country	GenBank Accession #	Start position	End position	Identity level, %
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	Spain	KJ701031	32242	32630	99.23
Helicoverpa SNPV AC53	*Helicoverpa* sp.	Australia	KU738904	32173	32561	98.97
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	India	KT013224	14454	14842	98.97
Helicoverpa zea single nucleopolyhedrovirus	*Helicoverpa armigera*	Brazil	KM596835	31027	31415	98.97
Helicoverpa zea single nucleopolyhedrovirus	*Helicoverpa zea*	Uzbekistan*	KJ004000	32226	32614	98.97
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	India	KM357512	1895	2283	99.23
Helicoverpa armigera SNPV	*Helicoverpa armigera*	Australia	KJ922128	32207	32595	98.97
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	South Africa	AY118080	460	848	98.97
Helicoverpa armigera nucleopolyhedrovirus	*Heliothis peltigera*	Turkey	MK507817	32100	32488	98.46
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	China	MT810812	32536	32924	98.46
Helicoverpa assulta nucleopolyhedrovirus	*Helicoverpa assulta*	China	MG569706	32470	32858	98.20
Hyblaea puera nucleopolyhedrovirus	*Hyblaea puera*	India	MH254887	29	341	98.72

* according to Kolosov A.V., personal communication.

Таблица 4. Доступные в GenBank нуклеотидные последовательности гена *lef8* изолятов вируса ядерного полиэдроза *Helicoverpa armigera*, использованные для сравнительного анализа в настоящей работе

Вид, изолят	Хозяин	Страна	Номер доступа в GenBank	Начальная позиция	Конечная позиция	Уровень сходства, %
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	Испания	KJ701031	32242	32630	99.23
Helicoverpa SNPV AC53	*Helicoverpa* sp.	Австралия	KU738904	32173	32561	98.97
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	Индия	KT013224	14454	14842	98.97
Helicoverpa zea single nucleopolyhedrovirus	*Helicoverpa armigera*	Бразилия	KM596835	31027	31415	98.97
Helicoverpa zea single nucleopolyhedrovirus	*Helicoverpa zea*	Узбекистан*	KJ004000	32226	32614	98.97
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	Индия	KM357512	1895	2283	99.23
Helicoverpa armigera SNPV	*Helicoverpa armigera*	Австралия	KJ922128	32207	32595	98.97
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	Южная Африка	AY118080	460	848	98.97
Helicoverpa armigera nucleopolyhedrovirus	*Heliothis peltigera*	Турция	MK507817	32100	32488	98.46
Helicoverpa armigera nucleopolyhedrovirus	*Helicoverpa armigera*	Китай	MT810812	32536	32924	98.46
Helicoverpa assulta nucleopolyhedrovirus	*Helicoverpa assulta*	Китай	MG569706	32470	32858	98.20
Hyblaea puera nucleopolyhedrovirus	*Hyblaea puera*	Индия	MH254887	29	341	98.72

* Согласно Колосову А.В., личное сообщение.

numerous unidentified isolates from lepidopterans belonging to different families (Table 6). The *rpb1* sequence, deposited in GenBank under the number ON099402, showed similarity with homologous *N. bombycis* sequences at the level of 96–99%, while similarity to other closely related species was 93% for *N. disstriae* (# HQ457438), 92% for *N. fumiferanae* (# HQ457435) and 91% for *N. pyrausta* (# MG182018). Spores isolated from the infected cotton bollworm larva measured 3.2–4.5(mean 3.9) × 2.0–2.7(mean 2.4) μm (n=12) and *N. bombycis* spores from silkworm – 3.8–4.4(mean 4.0) × 2.2–2.8(mean 2.4) μm (n=11).

Discussion

Baculoviruses and microsporidia are widely distributed in nature, and their detection in populations of the cotton bollworm is quite expected. In addition, since bacteria of the genus of *Wolbachia* are also widespread among Lepidoptera, it was expected to detect their presence in the studied samples of *H. armigera*. Yet, no *Wolbachia* was found. This can be due to the low frequency of this endosymbiont, as well as due to its uneven spatial and temporal distribution in local populations of the pest. In particular, though no published data in scientific literature were found concerning *Wolbachia* in the cotton bollworm, presence of respective GenBank entries indirectly indicate occasional detection of this endosymbiont in this host in India (# KY781914) and China (## EU399644 and EU753172).

Since the species diversity of baculoviruses in cotton bollworms over the vast territory of Russia had been practically unexplored at the beginning of the work, diagnostics was aimed at detecting baculovirus infections using degenerate primers, because of high evolutionary lability of viral genomes (Herniou et al., 2004). The virus isolates were found in only one geographic location, and the sequences of all of them were identical to each other and showed the maximum similarity to the HearNPV and HzNPV entries available in GenBank, with only minor genetic differences. Unfortunately, sequencing of the *lef8* locus had insufficient resolution to differentiate these species and, accordingly, to accurately diagnose the new isolates. This goal should therefore recruit analysis of other, more polymorphic loci (protein kinase, DNA polymerase, DNA helicase, chitinase, zinc finger protein, etc.) or whole genome sequencing. In addition, it is possible that the two indicated above species of the virus should rather be considered as intraspecific isolates, since they cross-infect

Table 5. Results of BLAST analysis of the complete genome of the HS-18 strain against the *Helicoverpa zea* nucleopolyhedrovirus (gray background) and *Helicoverpa armigera* nucleopolyedrovirus sequences

Таблица 5. Результаты BLAST-анализа полного генома штамма ХС-18 относительно сиквенсов *Helicoverpa zea* nucleopolyhedrovirus (серый фон) и *Helicoverpa armigera* nucleopolyhedrovirus

Species, strain Вид, изолят	GenBank Accession # Номер доступа в GenBank	Identity level, % Уровень сходства, %
Helicoverpa zea single nucleopolyhedrovirus	KJ004000	100.00
Helicoverpa zea single nucleopolyhedrovirus	AF334030	99.97
Helicoverpa SNPV AC53	KM596835	99.96
Helicoverpa armigera SNPV	KJ909666	99.55
Helicoverpa SNPV AC53	KJ922128	99.54
Helicoverpa SNPV AC53	KU738896	99.23
Helicoverpa SNPV AC53	KU738904	99.20
Helicoverpa SNPV AC53	KU738901	99.20
Helicoverpa SNPV AC53	KU738899	99.20
Helicoverpa SNPV AC53	KU738902	99.20
Helicoverpa SNPV AC53	KU738900	99.20
Helicoverpa SNPV AC53	KU738897	99.20
Helicoverpa SNPV AC53	KU738898	99.19
Helicoverpa armigera NPV NNg1	KU738903	99.13
Helicoverpa armigera nucleopolyhedrovirus	AP010907	99.00
Helicoverpa armigera nucleopolyhedrovirus	KJ701029	98.84
Helicoverpa armigera nucleopolyhedrovirus	KJ701033	99.20
Helicoverpa armigera nucleopolyhedrovirus	KJ701032	99.11
Helicoverpa armigera nucleopolyhedrovirus	KJ701030	99.01
Helicoverpa armigera NPV strain Australia	KJ701031	99.00
Helicoverpa armigera nucleopolyhedrovirus G4	JN584482	98.90
Helicoverpa armigera nucleopolyhedrovirus	AF271059	98.78
Helicoverpa zea single nucleopolyhedrovirus	AF303045	98.44

Table 6. GenBank entries of microsporidia isolates showing 100 % identity of small subunit ribosomal RNA sequence to the microsporidium from *Helicoverpa armigera* identified in the present study

Таблица 6. Записи в GenBank для изолятов микроспоридий, демонстрирующие 100 % идентичность последовательности малой субъединицы рибосомной РНК с микроспоридией из *Helicoverpa armigera*, выявленной в настоящем исследовании

Species, isolate Вид, изолят	Host species Вид хозяина	Country Страна	GenBank Accession # Номер доступа в GenBank
Nosema bombycis	*Bombyx mori*	Japan	AB125665
Nosema bombycis	*Antheraea mylitta*	India	AB036052
Nosema bombycis	*Bombyx mori*	Japan	AY259631
Nosema bombycis	*Bombyx mori*	No data	EU864525
Nosema bombycis (Nosema heliothidis)	*Helicoverpa armigera*	China	FJ772435
Nosema bombycis (Nosema spodopterae)	*Spodoptera litura*	Taiwan	AY747307
Nosema bombycis GD 1	*Bombyx mori*	China	JF443582
Nosema bombycis GNB3	*Bombyx mori*	China	MT510128
Nosema bombycis GX 1	*Bombyx mori*	China	JF443577
Nosema bombycis Sd-NU-IW8401	*Spodoptera depravata*	Japan	D85504
Nosema bombycis SES-NU	*Bombyx mori*	Japan	D85503
Nosema sp. C01	*Pieris rapae*	South Korea	AY383655
Nosema sp. CmM1	*Cnaphalocrocis medinalis*	China	KC836091
Nosema sp. CP JX-2014	*Catopsilia pyranthe*	China	KM001609
Nosema sp. *Hyblaea puera* 1	*Hyblaea puera*	India	GQ244502
Nosema sp. AA1	*Antheraea assamensis*	India	MG584870
Nosema sp. OSL-2014-3	*Spodoptera litura*	Japan	LC422302
Nosema sp. PM-1	*Papilio machaon Linnaeus*	China	KM190863
Nosema sp. PX1	*Plutella xylostellae*	Taiwan	AY960986
Nosema sp. *'S. litura'*	*Spodoptera litura*	Taiwan	AF238239
Nosema sp. TWSL-2014-1	*Spodoptera litura*	Taiwan	LC422303
Nosema sp. VSl-2007-13	*Spodoptera litura*	Viet Nam	AB569602
Nosema sp. YGSL-2015-2	*Spodoptera litura*	Japan	LC422315
Nosema sp. YY-2018a	*Athetis lepigone*	China	MF150255

American cotton bollworms and the cotton bollworms, the closely related insect species. In addition, levels of genetic divergence between viral isolates are extremely low, even when comparing among genome-wide sequences, (Kolosov et al., 2017). Detection of viruses with the same *lef8* haplotype in the phylogenetically distant species of *H. puera*, registered in GenBank, is interesting. However, the host identification requires additional verification, since infection of distantly related host species does not correspond to modern ideas about the species specificity of baculoviruses (Thiem, 1997; Song et al., 2016).

As for microsporidia, the range of their potential hosts is much wider. In particular, *N. bombycis* was isolated from various Lepidoptera, including representatives of the Noctuidae family (Iwano and Ishihara, 1991; Tokarev et al., 2020). The *rpb1* sequence of the new isolate was identical to the GenBank entry for *N. bombycis*, and its morphometric characteristics coincided with those of *N. bombycis*, which allows us to consider the microsporidia from the cotton bollworm as an isolate of this species. This corresponds to the wide range of hosts of this microsporidium confirmed by molecular genetic analysis of the natural *N. bombycis* infections in different species of Lepidoptera (Tokarev et al., 2020).

Interpreting low levels of occurrence of microsporidia and baculoviruses in local populations of the cotton bollworm, one should take into account the fact that the analyzed samples were collected over a limited period of time, during the pest outbreak in 2018–2020. An increase in prevalence of microsporidia and viruses corresponding to the growth of population density beneficial to horizontal transmission, has been recorded for various Lepidoptera species including *L. dispar* (Solter et al., 2010), *Choristoneura pinus* (van Frankenhuyzen et al., 2011), *Tortrix viridana* (Lipa, Madziara-Borusiewicz, 1976), *Taragama siva* (Ahmed, Kumar, 1998), *S. exempta* (Odindo, 1983). It could be presumed that in the case of the cotton bollworm, pathogens' prevalence levels do not increase during the outbreaks. This can be explained by high motility of larvae that helps to avoid overcrowding and cannibalism. Interestingly, cannibalism was repeatedly reported under laboratory conditions but never observed in the field (Dhandapani et al., 1993; Kakimoto et al., 2003; Zalucki et al., 2021). We hope that further studies will clarify the interactions between the prevalence of infections with endosymbionts and dynamics of *Helicoverpa armigera* population density.

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Полнотекстовая статья

МОЛЕКУЛЯРНАЯ ДИАГНОСТИКА ЭНДОСИМБИОНТОВ В ПОПУЛЯЦИЯХ ХЛОПКОВОЙ СОВКИ *HELICOVERPA ARMIGERA* (LEPIDOPTERA: NOCTUIDAE) В ЕВРОПЕЙСКОЙ ЧАСТИ РОССИИ

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Хлопковая совка *Helicoverpa armigera* – один из самых многоядных и космополитичных видов фитофагов. Внутриклеточные эндосимбионты широко распространены в популяциях чешуекрылых насекомых и часто имеют важное значение в их динамике численности. Данные о распространении энтомопатогенов у хлопковой совки на территории России в современных условиях практически отсутствуют. Гусеницы и имаго хлопковой совки были собраны в 2018–2020 гг. в Краснодарском крае, Воронежской и Саратовской областях и проанализированы методом ПЦР с использованием наборов группо-специфичных праймеров на бакуловирусы (локус *lef8*), бактерий рода *Wolbachia* (локус *wsp*) и микроспоридий (локус SSU rRNA) в количестве от 131 до 170 особей для разных групп патогенов. Положительная реакция на бакуловирусы отмечалась на уровне 16% для выборки из 32 особей Темрюкского района Краснодарского края 2018 г. Общая зараженность для всей выборки из 170 особей составила 2.9%. Обнаружено сходство нуклеотидной последовательности *lef8* на уровне 98.7–99.6% с изолятами вирусов ядерного полиэдроза хлопковой совки и американской хлопковой совки. Результаты тестирования выборки из 131 особей на присутствие бактерий рода *Wolbachia* были отрицательными. При ПЦР-скрининге на микроспоридий получен один положительный сигнал для выборки из 19 особей Красноармейского района Краснодарского края 2019 г., что соответствует 5%. Для всей выборки из 168 проанализированных особей зараженность составила 0.6%. Нуклеотидные последовательности фрагментов генов, кодирующих SSU рРНК и большую субъединицу РНК-полимеразы II, позволило идентифицировать новый изолят как *N. bombycis*.

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Ключевые слова: облигатные внутриклеточные паразиты, энтомопатогенные микроорганизмы, вредные чешуекрылые, естественная зараженность, вирус ядерного полиэдроза, Microsporidia, *Wolbachia*, *Nosema bombycis*

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