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Short communication

SUSCEPTIBILITY OF *BOMBYX MORI* LARVAE TO MICROSPORIDIA *NOSEMA BOMBYCIS* FROM THE SILKWORM AND *NOSEMA* SP. FROM THE COTTON BOLLWORM

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Microsporidia are widespread parasites and cause diseases in economically important insects. A microsporidian isolate NspHA22 was discovered in the cotton bollworm *Helicoverpa armigera* in South-Western Russia. It showed 100% sequence identity of small subunit rRNA gene to *Nosema bombycis,* a natural parasite of the silkworm *Bombyx mori.* However, after feeding second or third instar *B. mori* larvae with spores of the new isolate, insect mortality didn't differ from that of the control, and no sporulation was revealed in alive and perished insects. In contrast, feeding *N. bombycis* spores isolated from *B. mori* resulted in high levels of host mortality and intense parasite sporulation at all the infection dose and larval instars used. This likely indicates that the isolate NspHA22 belongs to a species different from *N. bombycis,* in spite of identity of rDNA sequences.

Keywords: parasitic protists, host range, virulence, laboratory culture, biological control

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Introduction

Microsporidia are parasitic protists that have a common origin with fungi (Bass et al., 2018). Many species of microsporidia are highly pathogenic for arthropods and significantly affect their populations (Becnel and Andreadis, 2014; LeBrun et al., 2022). Microsporidia belonging to several genera are known to regulate population dynamics of their host (Andreadis et al., 1996; Malysh et al., 2013, 2018). Practical interest in studying these parasites is due to their ability to actively multiply and cause a disease (microsporidiosis). There are many examples demonstrating the role of microsporidia in regulation of abundance of mass lepidopteran species (Issi, 1986; Frolov et al., 2008; Kermani et al., 2013; Simoes et al., 2015; Hopper et al., 2016). Particularly, the species of the genus *Nosema* might be devastating for insect populations, like the type species, *Nosema bombycis* Nägeli 1857, a natural parasite of *Bombyx mori* Linnaeus, 1758 (Bombycoidea: Bombycidae) that has been a threat to silk industry since ancient times (Bhat et al., 2009).

In 2019, a novel isolate was found in a population of cotton bollworms *Helicoverpa armigera* (Hübner 1808). Comparison of SSUrDNA fragments of this isolate with the orthologs of other representatives of the genus *Nosema* suggested identity or very close relationships of the isolated microsporidium to *N. bombycis* (Kononchuk et al., 2021). Due to similarity of the spore structure and low sequence divergence of rRNA genes among *Nosema* spp., differentiation of species within this group poses a serious problem (Kyei-Poku et al., 2008; Issi et al., 2020; Tokarev et al., 2020). The aim of this work is to evaluate the ability of the new isolate to infect the silkworm *Bombyx mori*, a type host of *Nosema bombycis*.

Materials and methods

Silkworm eggs were obtained from the Stavropol sericulture station (Pyatigorsk) and propagated at the facilities of All-Russian Institute of Plant Protection in St. Petersburg.

Spores of *N. bombycis*, isolate NbBM23, were produced in silkworms at the Scientific Research Institute of Sericulture (Tashkent, Uzbekistan) and propagated in the larvae of the beet webworm *Loxostege sticticalis* in St. Petersburg. The same approach was applied for mass production of the spores of the novel microsporidium, of the genus *Nosema*, named NspHA22, isolated from bollworm larvae collected in the Krasnodar region in 2022.

Total DNA was extracted using a simplified protocol of Sambrook et al. (1989) with modifications (Malysh et al., 2019). For DNA amplification, the primers targeting two loci were used. One locus was the small subunit ribosomal RNA (SSU rRNA) and the primers were 18f:1047r, annealing temperature (Ta) 54 °C, product size 900 bp (Weiss, Vossbrinck, 1999). Another locus was large subunit RNA polymerase (RPB1). Primers for this locus were designed specifically for this study, including the pairs a) nosRPB1for1 (GATCTYGCYTACAGTASAC), nosRPB1rev1 (AGCRGTGAGWGTATCTT), Ta = 52 °C, and b) nosRPB1for2 (GTTCAAGATACWCTCACYGGT), nosRPB1rev2 (AGRGTATCHGAATCDGC), Ta = 56 °C, producing amplicons of 900 and 600 bp, respectively. For PCR analysis we used DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) with the following cycling conditions: initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 1 min, annealing 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). The purified

amplicons were sequenced at Evrogen (Moscow) by a standard method of chain termination (Sanger, 1977). Sequence reads were processed with BioEdit (Hall, 1999).

Fresh spores of NbBM23 and NspHA22 were used to infect second instar larvae of the silkworm at the dosages of 10^3, 10^4 and 10^5 spores per individual using 3 t o5 replicates, 10 larvae per repetition. An aliquot of the spore suspension (100 µl) was applied to young mulberry leaves, which were placed on a moistened cotton wool and exposed to the second instar larvae. For the third instar larvae, a suspension with spores (200 µl) was applied to a mulberry leaf with its petiole inserted in a 5 ml glass vial filled with water and sealed with parafilm. The experimental variants included dosages of 10^4 and 10^5 spores/larvae. Each variant has 2 repetitions. Each repetition contained 40 larvae. In control, insects were treated similarly, but without addition of spores.

The experiments were carried out at the temperature of + 24 °C. Mortality was recorded daily for 60 days. Distilled water was used as a control. Insect corpses were homogenized and examined under the light microscope Carl Zeiss Imager M1 and Lab.A1, equipped with AxioCam video cameras and AxioVision software, in a bright field at magnifications of 400x and 1000x (oil immersion).

Smears showing no spores at all were considered negative while smears showing 1–10, 10–100 and >100 spores per microscope field at 40x magnification were scored as low ("+"), moderate ("++") and high intensity infection ("+++"), respectively.

Data processing was carried out using the Sigmaplot program (Systat Software, Inc.) using analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test.

Results and Discussion

The microsporidian isolate from the cotton bollworm showed 100 % identity to the previously characterized isolate from the same host and location, sampled two years earlier (Kononchuk et al., 2021). When compared to other Genbank-accessible entries, it also showed a high level of sequence correspondence of the diagnostic DNA fragments to those of the known species. In particular, the sequence of SSU rRNA was 100 % identical to those of several isolates of *N. bombycis* from GenBank, accession ## FJ772435, AY747307, D85504, and D85503. The isolates of *Nosema rachiplusiae* NRnuBA (# KY126433), *Nosema pyrausta* (# HM566196), *Nosema trichoplusiae* ATCC 30702 (# U09282), *Nosema (Vairimorpha) ceraces* (# EU267796), *Nosema tyriae* (# AJ012606), *Nosema (Vairimorpha) imperfect* (# AJ131645), *Nosema mylitta* Nm15SSU (# MN542655), *Nosema disstriae* (# EU219085), *Nosema antheraeae* (# EU864526), and *Nosema fumiferanae* (# EU219083) showed 99.4–99.7 % identity to *N. bombycis* and NspHA22. Less than 97.7 % similarity was found when compared to the following isolates: *Nosema granulosis* (# AJ011833), *Nosema (Vairimorpha) austropotamobii* (# MF344634), *Nosema empoascae* (# DQ996238), and *Nosema (Vairimorpha) cheracis* (# AF327408).

Fragments of the largest subunit RNA polymerase gene at two independently amplified loci showed similarity of about 97–98 % with other species of the genus *Nosema*, including *N. bombycis*, *N. trichoplusiae*, and for one of the loci the similarity was 97.8 % (Table 1). Similar levels of identity were found when these taxa were compared between each other.

Within 60 days after infection with NbBM23 spores, high mortality levels of larvae were observed in all variants. In particular, infection with 10^4 and 10^5 spores/larva resulted in 100 % mortality of 3rd instars on the 50th day (Fig. 1C). Moreover, death of insects infected with the maximal dosage started from the first week post infection. Similarly, 97–100 % mortality was observed on the 60th day in the experiment with 2nd instars (Fig. 1B), and starting from the first week after infection, the death rate did not differ significantly between the maximal and average dosages.

As many as 97–99 % of larvae fed with NbMB23 spores at the three dosages became heavily infected. Fresh smears from these insects contained numerous spores. In the cases of infections with 10^4 and 10^5 spores per larvae, the intensity was high (>100 spores per microscope field). Only in the case of the lower dose (10^3 spores/larva) the intensity was occasionally lower (1–10 spores per field).

On the contrary, when infected with NspHA22, most larvae developed successfully (Fig. 1A) and no significant differences were found between the variants of the infection

Table 1. Results of BLAST analysis for microsporidia isolates of largest subunit RNA polymerase sequences available in Genbank with the microsporidium from *Helicoverpa armigera* identified in the present study

Таблица 1. Результаты BLAST-анализа изолятов микроспоридий с последовательностями малой субъединицы РНК-полимеразы, доступных в Genbank, с изолятом микроспоридии *Helicoverpa armigera*, идентифицированными в настоящем исследовании

Species, isolate Вид, изолят	Country Страна	GenBank Accession # Номер доступа в GenBank	Identity levels, % Уровни идентичности, % (fragments sequenced with primers NosRPB1)	
			For1/rev1	For2/rev2
Nosema bombycis	UK	JX213755	96.6	97.76
Nosema bombycis	China	JX213753	96.94	96.26
Nosema bombycis	UK	DQ996231	96.81	96.26
Nosema trichoplusiae	UK	DQ996234	97.06	97.26
Nosema tyriae	UK	AJ278948	95.70	97.51
Nosema disstriae	Canada	HQ457438	93.80	94.24
Nosema antheraeae	China	HQ215550	-	93.44
Nosema fumiferanae	Canada	HQ457435	94.34	91.98
Nosema pyrausta	Russia	MG182018	91.69	-

and the control. Microscopy did not detect spores in larvae of all instars during the whole period of the experiment (Table 2). According to the literature, *B. mori* id susceptible to different species of microsporidia (Kawakami at al., 1994; Kawarabata, 2003). The cotton bollworm isolate, however, was not capable of infecting the silkworm at any of the dosages tested, unlike *N. bombycis*. This observation contradicts to the assumption that NspHA22 belongs to *N. bombycis,* as any isolate of this species is expected to be infective and virulent to the type host.

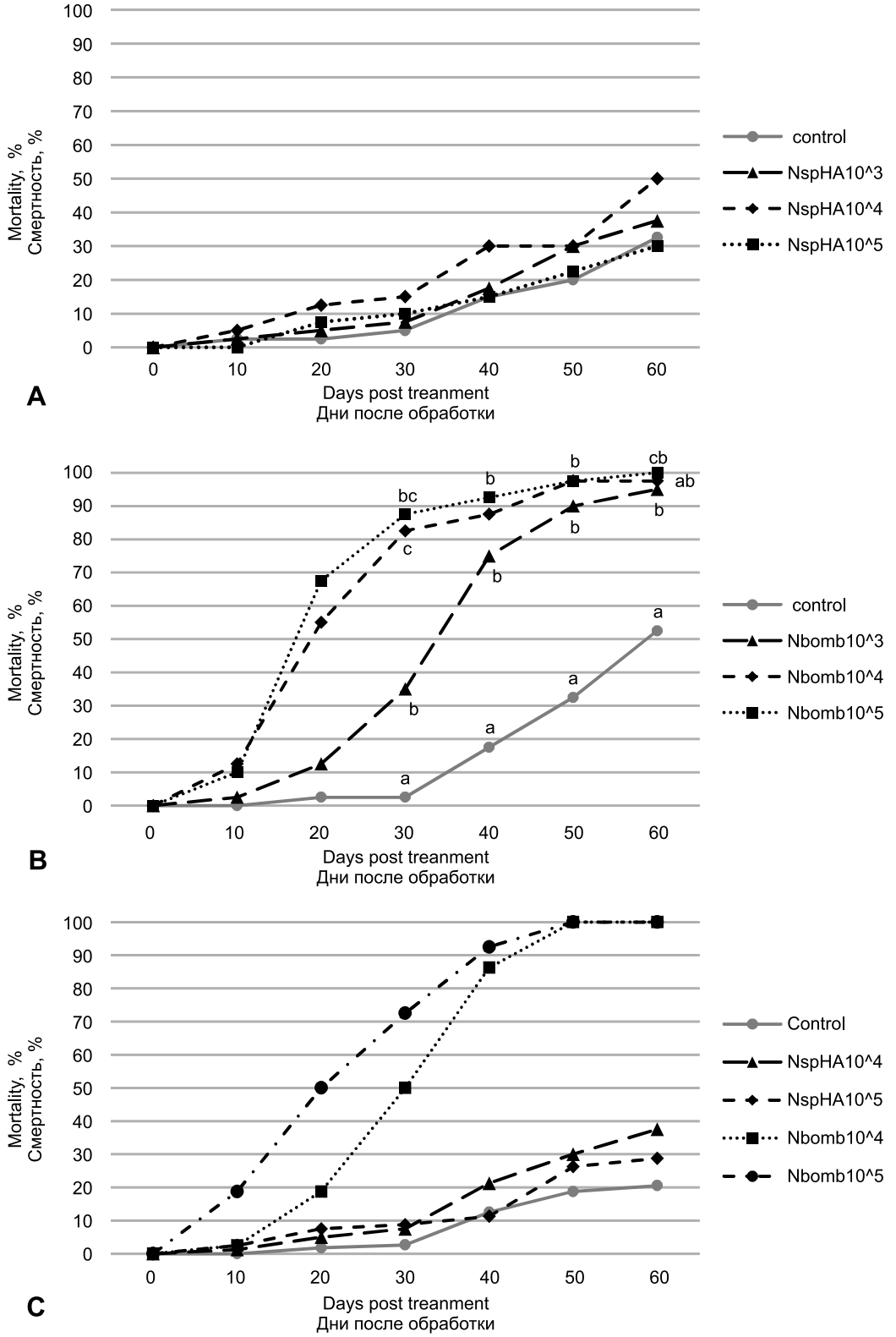

Figure 1. Dynamics of silkworm mortality as a result of feeding II (**A, B**) or III instar larvae (**C**) with two isolates of microsporidia from the cotton bollworm NspHA (**A, C**) and the silkworm NbBM (**B, C**). Control without treatment with entomopathogens

Рисунок 1. Динамика смертности тутового шелкопряда в результате скармливания личинкам II (**A, B**) или III возраста (**C**) двух изолятов микроспоридий – из хлопковой совки NspHA22 (**A, C**) и из тутового шелкопряда NbBM23 (**B, C**). Контроль без обработки энтомопатогенами

Table 2. Data on microscopy of silkworm larvae infected with microsporidia NbBM23 and NspHA22
Таблица 2. Данные микроскопии тутового шелкопряда, зараженного изолятами микроспоридий NbBM23 и NspHA22

Species, isolate Вид, изолят	Dosage, spores per larva Дозировка, спор на гусеницу	Sample size, pcs Объем выборки, шт.	Infection prevalence level, % Зараженность, %	Infection intensity (from …. to) Интенсивность заражения (от …. до)
Nosema bombycis NbBM23	10^3	34	97	+ …. +++
	10^4	76	98.7	++ …. +++
	10^5	92	99	++ … +++
Nosema sp. NspHA22	10^3	18	0	-
	10^4	40	0	-
	10^5	20	0	-

Infection intensity is defined as: (-) negative, no infection; (+) low, (++) medium, (+++) high.

As for the studies at the molecular level, unfortunately, sequencing of the SSU rRNA and RPB1 loci did not make it possible to accurately identify the new isolate and differentiate it from the previously described species. As shown by recent studies, there are microsporidian taxa with high levels of SSU rRNA sequence similarity, yet referred to as different species, e.g. *N. bombycis* vs *N. pyrausta* (Tokarev et al., 2015), *Vairimorpha (Nosema) lymantriae* vs *V. disparis* (Tokarev et al., 2020), and *Tubulinosema loxostegi* vs *T. acridophagus* (Malysh et al., 2013). In order to reliably differentiate species within the *Nosema-Vairimorpha* lineage at the molecular level, it is probably necessary to analyze other more polymorphic loci (protein kinase, DNA helicase, chitinase, zinc finger protein, etc.) or the whole genome sequence data.

Meanwhile, the ability or failure to infect a particular host insect, such as the type host of *N. bombycis,* can be relied upon as a complimentary taxonomic character, given that it is testified using a solid number of bioassays, like in the present study.

Conclusion

The silkworm as a type host of *N. bombycis* is highly susceptible to this microsporidium and shows high levels of infection from the first week of the experiment. Meanwhile, the isolate from the cotton bollworm does not show an ability to infect the silkworm larvae. This indirectly indicates that these two isolates belong to different species of microsporidia.

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Краткое сообщение

ВОСПРИИМЧИВОСТЬ ГУСЕНИЦ *BOMBYX MORI* К МИКРОСПОРИДИЯМ *NOSEMA BOMBYCIS* ИЗ ТУТОВОГО ШЕЛКОПРЯДА И *NOSEMA* SP. ИЗ ХЛОПКОВОЙ СОВКИ

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Микроспоридии относятся к широко распространенным паразитам и вызывают заболевания экономически важных насекомых. Изолят микроспоридии NspHA22 обнаружен у хлопковой совки *Helicoverpa armigera* на Юго-Западе России. Он показал 100 %-ную идентичность последовательности гена малой субъединицы рРНК с *Nosema bombycis*, естественным паразитом тутового шелкопряда *Bombyx mori*. Однако после скармливания гусеницам второго и третьего возраста *B. mori* спор нового изолята смертность насекомых не отличалась от контроля, образования спор не выявлено. Напротив, заражение спорами *N. bombycis* из тутового шелкопряда вызывало высокую смертность хозяина и интенсивное спорообразование паразита при всех использованных дозах заражения и возрастах гусениц. Это, вероятно, указывает на то, что изолят NspHA22 принадлежит к другому виду, отличному от *N. bombycis*, несмотря на идентичность последовательностей рДНК.

Ключевые слова: паразитические протисты, круг хозяев, вирулентность, лабораторная культура, биологическая борьба

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