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Morphological and genetic diversity of microsporidia infecting beet webworm *Loxostege sticticalis* (L.) (Pyraloidea, Crambidae) in Russia

Морфологическое и генетическое разнообразие микроспоридий лугового мотылька *Loxostege sticticalis* (L.) (Pyraloidea, Crambidae) фауны России

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Ключевые слова: микроспоридии, луговой мотылёк, биоразнообразие, молекулярная филогения, *Vairimorpha thomsoni* comb.n.

Abstract. Microsporidia are ubiquitous parasites of animals, being most abundant in insects. Microsporidian spores of three district morphotypes were recovered from beet webworm sampled in Krasnodar territory (KT) in 2006, in Novosibirsk region (NR) in 2010 and in Irkutsk Region (IR) in 1982. The microsporidium from KT possessed elongated oval diplokaryotic spores and a ribosomal RNA (rRNA) gene sequence displaying 95 % similarity to that of the species belonging to the genus of *Endoreticulatus.* The microsporidium from NR possessed broadly oval diplokaryotic spores and its rRNA gene sequence suggested its allocation to the genus *Tubulinosema* (with 98.5–99.4 % sequence similarity to other representatives of this genus). Finally, the microsporidium from IR possessed monokaryotic spores in sprophorous vesicles by 8 (octospores); however, its rRNA gene sequence was identical (100 %) to that of *Nosema thomsoni*, but highly similar to *Vairimorpha* spp. (95.1–99.5 %). The latter genus incorporates the species with two sporogonies and observation of both *Nosema*-like spores (in the type host *Choristoneura conflictana*) and octospores (in the beet webworm), further supported by molecular phylogenetic study, claims for transfer of *N. thomsoni* to the genus *Vairimorpha* and the creation of a new combination, *Vairimorpha thomsoni* **comb.n.**

Резюме. Микроспоридии — широко распространённые паразиты животных, наиболее многочисленные у насекомых. Споры микроспоридий трёх различных морфотипов обнаружены в особях лугового мотылька, собранных в 2006 г. в Краснодарском крае (КК), в 2010 г. в Новосибирской области (НО) и в 1982 г. в Иркутской области (ИО). Микроспоридия из КК обладает удлинённо-овальными диплокариотическими спорами и сиквенсом гена рибосомальной РНК (рРНК), показавшим 95 %-ное сходство с таковыми представителей рода *Endoreticulatus.* Микроспоридия из НО имеет широко-овальные диплокариотические споры и сиквенс гена рРНК, сходный на 98,5–99,4 % с сиквенсами видов рода *Tubulinosema*, что предполагает её отнесение к этому роду. Наконец, микроспоридия из ИО обладает монокариотическими спорами в спорофорных пузырьках, содержащих по 8 спор (октоспоры). Однако, её нуклеотидная последовательность гена рРНК идентична таковой *Nosema thomsoni*, но при этом обладает высоким сходством с сиквенсами представителей рода *Vairimorpha* (95,1–99,5 %). Последний объединяет виды, имеющие два типа спорогонии, и обнаружение у *N. thomsoni* как *Nosema*-подобных спор (в типовом хозяине *Choristoneura conflictana*), так и октоспор (в луговом мотыльке), вкупе с данными молекулярно-филогенетического исследования, предполагает перевод *N. thomsoni* в род *Vairimorpha* с созданием нового таксона, *Vairimorpha thomsoni* **comb.n.**

Introduction

The beet webworm, *Loxostege sticticalis* (L., 1761) is a dangerous pest, as it causes serious damage to the crops such as soybean, sugar beet, alfalfa, sunflower and many others during its outbreaks in Eurasia, including Northern China and steppe zones of European and Asian parts of Russia [Chen Xiao et al., 2008; Frolov et al., 2008]. It also damages crops in North America [Pepper, 1938] and is referred to as an intro-

duced pest there [Peairs, Davidson, 1956]. In Russia, the recent outbreak of this pest began in 2008 in Eastern Siberia and the Far East. During the consequent three years, the outbreak was observed also in Western Siberia and European part of Russia, which is consistent with the high migratory capacity of the insect pest [Govorov et al., 2012]. Recent studies of the pest populations in the European part of Russia (Krasnodarskii Krai) showed that the beet webworm population densities are negatively correlated to the incidence rates of microsporidian infection of the previous generation of the host [Frolov et al., 2008]. This suggests possible involvement of the microsporidian parasites in regulation of its insect host dynamics.

Microsporidia are parasites of all the major taxa of animals, but most abundant in insects. Dozens of microsporidial species have been reported from the Lepidoptera, and the vast majority of these parasites belong to the genera *Nosema* Naegeli, 1857 [Tsai et al., 2003, 2009; Hylis et al., 2006; Johny et al., 2006; Kyei-Poku et al., 2008; Zhu et al., 2010; Guan et al., 2012] and *Vairimorpha* Pilley 1976 [Canning et al., 1999; Vávra et al., 2006; Wang et al., 2009; Liu et al., 2012]. Other genera that include species infecting Lepidoptera are *Cystosporogenes* Canning, Barker, Nicholas, Page, 1985 [Canning et al., 1985; Kleespies et al., 2003; Solter et al., 2010], *Endoreticulatus* Brooks, Becnel, Kennedy, 1988 [Cali, El Garhy 1991; Xu et al., 2012], *Orthosomella* Canning, Wigley, Barker, 1991 [Canning et al., 1991] and *Vavraia* (Weiser, 1947) Weiser, 1977 [Bourner et al., 1996].

Unfortunately, the species composition of microsporidia infecting the beet webworm populations within the pest area is poorly studied, which makes it difficult to interpret the role of distinct parasite species in the population dynamics of its insect host. In the present study, we combined light microscopy and DNA analysis to evaluate the diversity of microsporidia infecting beet webworm populations sampled across Russia.

Materials and methods

Adults of *L. sticticalis* were caught by net during the periods of mass flight in June 2006 in South-Western Russia, Krasnodarskii Krai (KT), Slavyanskii raion, and in July 2010 in Western Siberia, Novosibirskaya Oblast (NR), Karasukskii raion. The moths were stored as dry cadavers at room temperature (RT) or frozen at –20 °C. An infected larva collected in 1982 in Eastern Siberia, Irkutskaya Oblast (IR) was stored as a dry cadaver at RT. Smears were prepared from dry cadavers homogenized in a drop of water and examined using conventional light microscopy. The microsporidian spores were fixed and stained with diamidine phenylenindole [DAPI, Tokarev et al., 2007]. Three additional samples of microsporidian spores were used from *Agrotis segetum* Schiffermüller, 1775 (1 sample) and *Helicoverpa armigera* Hübner, 1827 (2 samples) collected in 2004 in Uzbekistan and stored in water suspension at +4 °C.

For molecular phylogenetic study, the infected specimens (or purified spores) were homogenized in 1.5 ml test tubes using an adapted Teflon pestle. The genomic DNA samples were extracted as described by Tokarev et al. [2010]. The partial small subunit (SSU) ribosomal RNA (rRNA) gene was amplified using the following sets of primers: 18f:530r, 18f:1047r or 18f:1492r. To span the internal transcribed spacer (ITS) and large subunit (LSU) rRNA gene region, the primer pair 1061f:ls580r was additionally used [Weiss, Vossbrinck, 1999]. PCR was run using a Bio–Rad MyCycler in 20 μL volume containing 10 μL DNA template, 2 μl of 10×PCR buffer, 1 μL of 10 μM dNTPs mixture, 1 μL of each forward and reverse 10 μM primers (Evrogen, Russia) and 1 U of Colored Taq-polymerase (Sileks, Russia). A first cycle of denaturation was carried out at 95 °C for 5 min, and a last cycle of extension was carried out at 72 °C for 10 min. Samples were amplified for 30 cycles of denaturation at 95 °C for 60 s, annealing at 54 °C for 30 s and elongation at 72 °C for 30 s (with 18f:530r primers) or 60 s (with other primer sets). For amplification with primers 18f:1492r, the number of PCR cycles was increased from 30 to 50 and 1 μl aliquot of the resulted PCR mix was used for a consequent PCR (reamplification) under the same conditions to obtain visible bands. The PCR products were gel purified, cloned into pAL–TA vector (Evrogen, Russia) and sequenced in both directions. The alignment of the newly obtained sequences with those showing significant similarity was done automatically using CLUSTAL W algorithm and edited by eye in BioEdit v7.0.8.0 [Hall, 1999]. Phylogenetic reconstructions were carried out with Bayesian Inference (BI) in MrBayes v3.1.2 [Ronquist, Huelsenbeck, 2003] and Maximum Likelihood (ML) in PAUP* v4.0 β 10 [Swofford, 2003].

Results and Discussion

Three distinct spore morphotypes were observed in three samples examined, respectively. The microsporidium from KT possessed elongated oval diplokaryotic spores (Fig. 1A). Genotyping with 18f:1047r primers produced a partial SSU rRNA gene sequence 853 bp long. It was identical by 95 % to *Endoreticulatus schubergi* (Zwölfer, 1927) Cali, El Ghary, 1991 from *Lymantria dispar* (L., 1759) and *Endoreticulatus bombycis* Pan, Wan, Lu, Zhou, Xiang, 2003 from *Bombyx mori* (L., 1758), respectively. Absence of ultrastructural data and the discrepancy between morphological characters, namely diplokaryotic spores in the microsporidium under investigation and monokaryotic spores typical of *Endoreticulatus* spp., didn't allow to place this parasite to the latter genus or to create a new taxon. Microsporidian spores of similar morphotype were described as *Nosema sticticalis* Issi, Simchuk, Radischeva, 1980 from beet webworm sampled in 1972 in Moldova [Issi et al., 1980], but absence of type material available for ultrastructural and molecular studies prevent us from correct identification of this parasite in the view of modern taxonomy of Microsporidia.

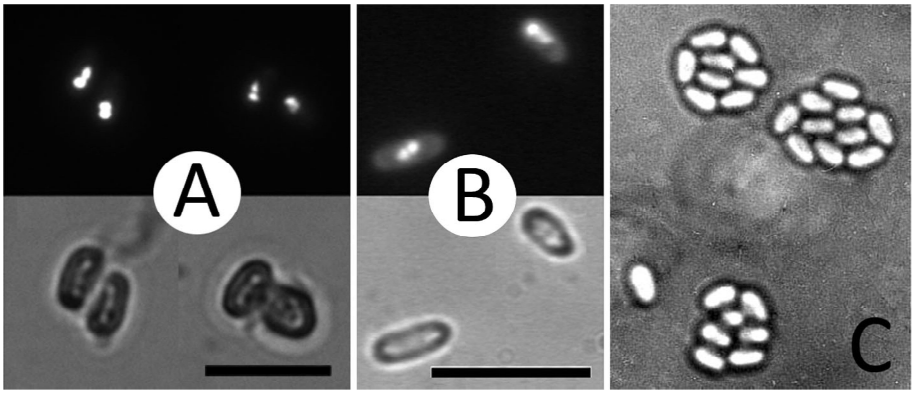

Fig. 1. Morphotypes of microsporidian spores isolated from beet webworm sampled in Krasnodar Territory in 2006 (A), in Novosibirsk region in 2010 (B) and in Irkutsk region in 1982 (C). A, B — spores fixed and stained with DAPI, upper and lower rows of images correspond to DAPI fluorescence and bright field, respectively; C — unfixed smear (archive photo). Scale bar 10 μm.

Рис. 1. Морфотипы спор микроспоридий, выделенных из лугового мотылька в Краснодарском крае в 2006 г. (A), в Новосибирской области в 2010 г. (B) и в Иркутской области в 1982 г. (C). A, B — споры фиксированные и окрашенные ДАФИ, верхний и нижний ряды изображений соответствуют флюоресценции ДАФИ и светлому полю; C — нефиксированный мазок (архивное фото). Масштабная линейка 10 мкм.

The microsporidium from NR possessed broadly oval diplokaryotic spores (Fig. 1B). For genomic DNA extraction, the infected insect tissue was either filtered with a 2 mL syringe through a cotton plug or used unfiltered. The PCR products were obtained from both filtered and unfiltered homogenates using 18f:530r primers. However, there was a remarkable difference between the unfiltered and filtered homogenate samples in term of the size of the major amplified band. In the unfiltered samples, there was a major band of *ca* 600 bp only. In the filtered samples, two bands were produced, *ca* 450 bp and *ca* 600 bp long, respectively (Fig. 2). The size of 600 bp does not correspond to the expected size of the microsporidia rRNA gene fragment amplified using 18f:530r primers. Its cloning and sequencing resulted in a sequence 549 bp long (Genbank accession # JQ906778) which showed *ca* 97 % similarity to the lepidopteran insect rRNA gene sequences and no significant similarity to the available microsporidian sequences. It is therefore identified as the rRNA gene sequence of the insect host, *L. sticticalis.* The filtration of the homogenate of the infected insect tissues thus decreases the non-specific amplification of insect 18S RNA gene fragment with primers 18f:530r. It can be explained by removal of large pieces of insect tissues during the filtration. The DNA extracted from the filtered homogenate was used for further molecular studies of the microsporidia, as it suggested the specific amplification of microsporidian rRNA gene fragment (band *ca* 450 bp long). Two amplicons *ca* 1400 and 1000 bp long, were amplified using 18f:1492r and ss1061f:ls580r primers. The two obtained sequences were identical in their expected region of overlapping and the resulted concatenated sequence was 1876 bp long (Genbank accession # JQ906779). BLAST analysis showed the maximal similarity of this partial SSU-ITS-LSU sequence to the rRNA gene of *Tubulinosema ratisbonensis* Franzen, Fischer, Schröder, Schölmerich, Schneuwly, 2005 from *Drosophila melanogaster* Meigen, 1830 and *Tubulinosema kingi* (Kramer, 1964) Franzen, Fischer, Schröder, Schölmerich, Schneuwly, 2005 from *Drosophila willinstoni* Sturtevant, 1916 of 98.4 % and 97.7 %, respectively. The SSU sequence of the new microsporidium, 1399 bp long, showed similarity of 99.4 % to *T. ratisbonensis* and *Tubulinosema acridophagus* (Henry, 1967) Franzen, Fischer, Schröder, Schölmerich, Schneuwly, 2005 from *Schistocerca americana* Drury, 1770*,* 99.3% to *Tubulinosema hippodamiae* Bjørnson, Le, Saito, Wang, 2011 from *Hippodamia convergens* Guérin-Méneville, 1842 and 98.5 % to *T. kingi*. The ITS sequence, 41 bp long, showed similarity of 83.3 % and 79.0 % to *T. ratisbonensis* and *T. kingi,* respectively. The partial LSU rRNA gene sequence of the new microsporidium, 436 bp long, is similar by 96.7 % to the respective sequences of in *T. ratisbonensis* and *T. kingi*, the two latter being iden-

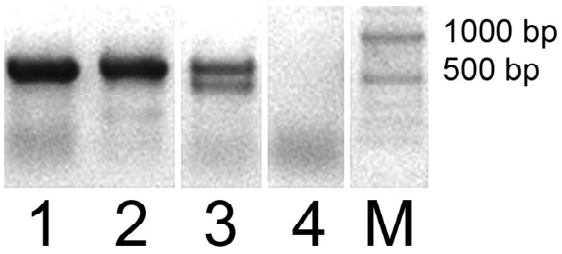

Fig. 2. Electrophoregram of DNA samples from the microsporidia-infected beet webworm adults after PCR with 18f:530r primers. 1, 2 — unfiltered homogenate; 3 — cotton-filtered homogenate; 4 — negative control (no DNA); M — a molecular weight marker.

Рис. 2. Электрофореграмма образцов ДНК из заражённых микроспоридиями имаго луговых мотыльков после ПЦР с праймерами 18f:530r. 1, 2 — нефильтрованный гомогенат; 3 — гомогенат, фильтрованный через вату; 4 — отрицательный контроль (без ДНК); M — маркёр молекулярного веса.

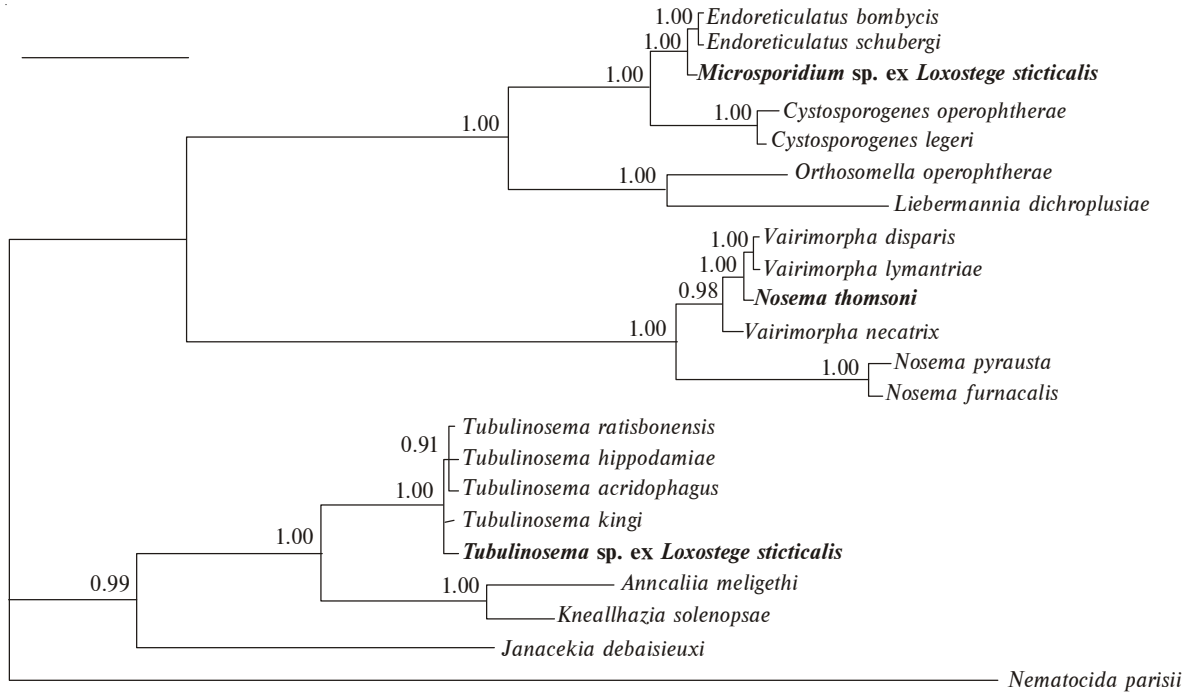

Fig. 3. Phylogram demonstrating polyphyletic nature of microsporidia isolated from the beet webworm in the present work (set in bold). Scale bar is 0.1 expected change per site.

Рис. 3. Филограмма, демонстрирующая полифилетическое происхождение микроспоридий, выделенных из лугового мотылька в настоящей работе (отмечены полужирным шрифтом). Масштабная линейка — 0,1 ожидаемой замены на сайт.

tical at the aligned region. Overall, the SSU rRNA gene sequence similarity between the distinct species of the genus of *Tubulinosema* ranged from 98.5 % (between the new microsporidium and *T. kingi*) to 99.6 % (between *T. acridophagus* and *T. ratisbonensis*).

The molecular phylogenetics suggests allocation of this new microsporidium to the genus of *Tubulinosema*, and ultrastructural features of this parasite, reported elsewhere [Malysh et al., in press] further confirm this proposition. This finding extends the host range of the *Tubulinosema* species to include Lepidoptera. Other insect host orders where microsporidia of this genus were found are Orthoptera, Diptera [Franzen et al., 2005; Issi et al., 2008] and Coleoptera [Bjørnson et al., 2011]. Moreover, the representatives of *Tubulinosema* from Orthoptera were found able to infect Lepidoptera [Krylova, Nurzhanov, 1989] and immunocompromised humans [Choudhary et al., 2011; Meissner et al., 2012].

The microsporidium from IR possessed octospores, i.e. monokaryotic spores in sprophorous vesicles by 8 (Fig. 1C). The DNA from this archive sample, stored over 20 years at RT, could be however amplified with the primer set 18f:1047r to produce a partial SSU rRNA gene 844 bp long. It was 100 % identical to the respective sequence of *Nosema thomsoni* Wilson, Burke, 1971 from *Choristoneura conflictana* Walker, 1863. The identical SSU rRNA sequence was obtained for the *Nosema*-like microsporidia from lepidopteran pests collected in Uzbekistan. It allows identification of all these isolates as *N. thomsoni* and extends its natural host range to *L. sticticalis*, *A. segetum* and *H. armigera*. Other closely related species were *Vairimorpha disparis* (Timofejeva, 1956) Vavra, Hylis, Vossbrinck, Pilarska, Linde, Weiser, McManus, Hoch, Solter, 2006 from *Lymantria dispar* (99.5 %) and its isolate known as «*Vairimorpha lymantriae*» (99.2 % sequence similarity), as well as *Vairimorpha necatrix* from *Pseudaletia unipuncta* (95.1 %). The diagnosis of the genus *Vairimorpha* includes two sporogonies with formation of two types of spores within one host [Canning, Vavra, 2000]. The first sporogony is so called *Nosema*-like (spores are single, diplokaryotic) while the second one is octosoporous, so called *Thelohania*-like (spores are in sporophorous vesicles by 8, monokaryotic). However, this is acknowledged that the two sporogonies may not be always displayed simultaneously. For example, in a continuous lab culture of *Galleria mellonella* F., 1798 infected with *Vairimorpha ephestiae* (Mattes, 1928) Weiser, Purrini, 1985 the octosporous sporogony was never observed [Vorontsova et al., 2004] and in a population of *Pieris brassicae* (L., 1758), infected with *Vairimorpha mesnili* (Paillot, 1918) Malone, McIvor, 1996, the octosporous sporogony was observed only once per four years [Issi, 1986]. The sequence similarity is therefore a more reliable taxonomic marker as compared to the life cycle observation and both approaches clearly show that *Nosema thomsoni* should be placed to the genus of *Vairimorpha* as a new combination of *Vairimorpha thomsoni* comb. nov.

The three microsporidia found in the beet webworm in the present study are therefore polyphyletic in

their origin (Fig. 3) and belong to the different lineages of parasites which are either associated with lepidopteran hosts (*Vairimorpha* and *Endoreticulatus*) or display a wider host range (*Tubulinosema*). The findings of *V. thomsoni* in Canada, Eastern Siberia and Uzbekistan seemingly reflects its comparatively wide host range and ability to infect hosts like *L. sticticalis*, characterized by Holarctic distribution. More species of microsporidia are expected being found when monitoring of *L. sticticalis* populations will be continued.

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