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Full-text Article

DETECTION OF MICROSPORIDIA INFECTING BEET WEBWORM *LOXOSTEGE STICTICALIS* (PYRALOIDEA: CRAMBIDAE) IN EUROPEAN PART OF RUSSIA IN 2006–2008

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The beet webworm *Loxostege sticticalis* (L.) is a major insect pest that causes serious damage of agricultural crops in Russia, China and adjacent countries. Microsporidia are obligate intracellular parasites that negatively affect population density of many insect hosts including Lepidoptera. In particular, infection with microsporidia is an important mortality factor for *L. sticticalis*. Special methodology for the identification of microsporidia associated with terrestrial insects is required. In the present paper we report the results of screening beet webworm moths for microsporidia using two techniques, i.e. light microscopy (LM) and PCR. Adult moths were sampled in 2006–2008 in the European part of Russia: Rostov Region, Krasnodar Territory and Republic of Bashkortostan. Microsporidia infections were detected in insects collected from all sampling sites. Examination of smears by LM showed presence of microsporidian spores in 3.4% of samples (N=98). PCR analysis of the same dataset was positive in 6.7% of samples, including those containing and not containing spores. The higher infection rate determined by PCR is likely connected with the fact that only mature spores

can be unequivocally identified by LM, whereas PCR also allows detection of otherdevelopmental stages of microsporidia. Partial sequencing of an amplicon from Krasnodar Territory showed its close relatedness to *Endoreticulatus poecilomonae* from *Poecilimon thoracicus* Fieber (Orthoptera: Tettigoniidae).

Key words: microsporidia, beet webworm, diagnostics, fluorescent microscopy, DAPI, PCR, rDNA, *Endoreticulatus*

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Introduction

The beet webworm *Loxostege (Pyrausta) sticticalis* L. is an important outbreak pest, causing serious damage to the crops such as soybean, sugar beet, alfalfa, sunflower and other crops in Eurasia, including Northern China and steppe zones of European and Asian parts of Russia (Chen Xiao et al., 2008; Frolov et al., 2008). Recent studies have shown that the insect population density correlated with the prevalence of microsporidian infection in the previous generation, suggesting that microsporidia is an important factor of the regulation of beet webworm populations in nature (Frolov et al., 2008). Different species of microsporidia are able to infect beet webworm, including *Nosema loxostegi* (Issi et al, 1980), *Tubulinosema* sp., *Nosema* sp., *Nosema ceranae* (Malysh et al., 2018), *Tubulinosema loxostegi* (Malysh et al., 2013b) and *Vairimorpha thomsoni* (Malysh et al., 2013a).

The criterion of microsporidian infection rate is included into the specified forecast model of this dangerous agricultural pest (Malysh, 2006), substantiating the need for a reliable, fast and sensitive technique for detection of microsporidian infection. Light microscopic observation of microsporidian spores in insect tissue samples may reflect only a part of the infected individuals (Sokolova et al., 2004), but this technique is important to reveal the infection at the sporogonial stage of the parasite's development. Molecular detection is more sensitive and provides specified data on parasite prevalence, species composition and genetic polymorphism.

In the present paper, we report the results of screening of *L. sticticalis* adults collected in 2006–2008 at three sample sites located in European part of Russia for microsporidia using light microscopy (LM) and PCR and demonstrate infection with microsporidia belonging to the genus *Endoreticulatus*.

Materials and Methods

Adult moths of beet webworm were caught by net at three sampling sites in the European part of Russia: (a) pastures in Salsk District, Rostov Region, in July 2006; (b) meadows in Slavyansk District, Krasnodar Territory, in July 2007 and (c) a sugar beet field in Ufa District, Republic of Bashkortostan in August 2008 (Fig. 1). Moth were stored dried or fixed in ethanol (samples from Ufa) for 2–3 months at room temperature prior to analysis. For light microscopy (LM), moths were homogenized individually in 50 µl of distilled water, smears were prepared and the homogenates were stored frozen at –22 °C for subsequent DNA extraction. Smears were examined using bright field light microscopy. The smears suspected for the presence of microsporidian spores were dried, fixed with methanol, stained with 5 µM aqueous diamidine phenylenindole (DAPI) solution and examined

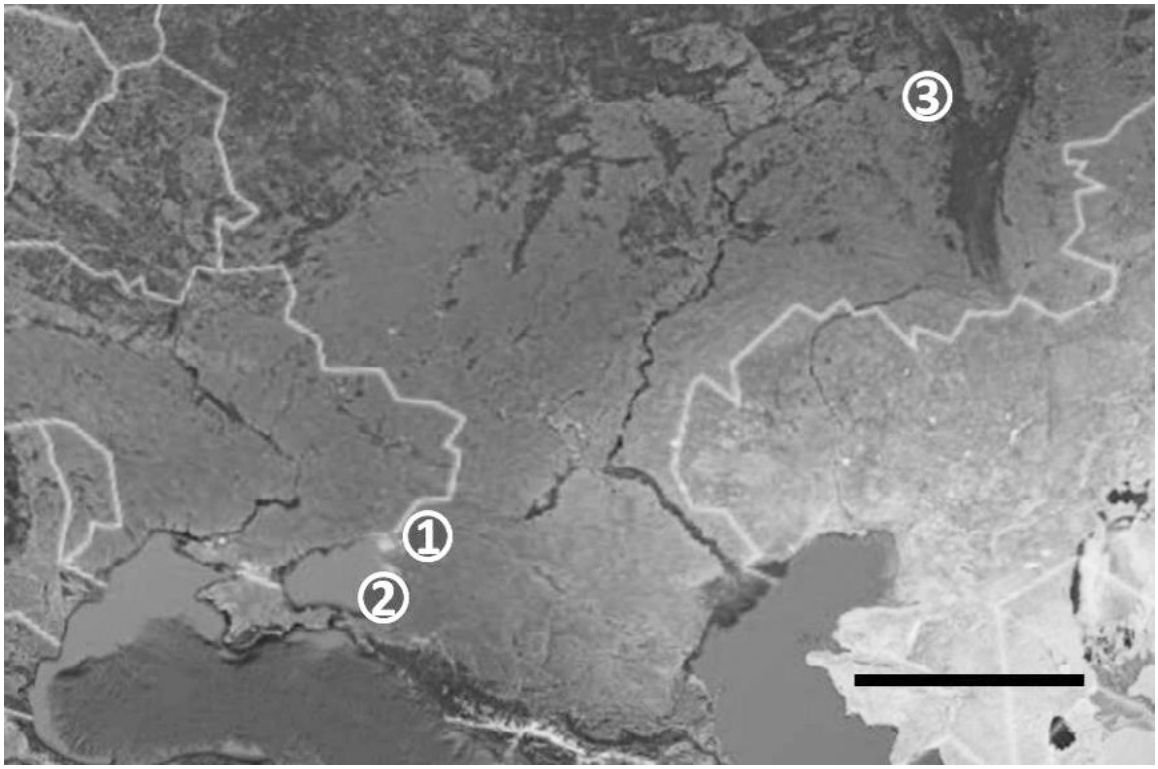

Figure 1. *Loxostege sticticalis* sampling sites: (1) Salsk District, Rostov Region, July 2006; (2) Slavyansk District, Krasnodar Territory, July 2007; (3) Ufa District, Republic of Bashkortostan August 2008

using Carl Zeiss Axioscope-2 equipped with epifluorescence and a digital camera (Tokarev et al., 2007).

For genomic DNA extraction, moth homogenates were re-homogenized with a plastic pestle in 1.5 mL microcentrifuge tubes in 100 µl lysis buffer, containing 2% CTAB, 1.4 M NaCl, 100 mM EDTA, 100 mM Tris-Cl (pH 8.0). After homogenization, 500 µl lysis buffer with 0.2% β-mercaptoethanol and 10 µl proteinase K (20 mg mL^{-1}) were added to the samples and incubated for 3 hrs at 65 °C. DNA was further extracted with phenol-chloroform, precipitated with isopropanol and washed with 70% ethanol (Sambrook et al., 1989). Dried DNA pellets were resuspended in 50 µl of deionized molecular grade water. PCR was run using a Bio-Rad iCycler in 10 µl volume containing 5 µl DNA template, PCR buffer, 0.25 mM dNTPs; 1 U Taq-polymerase (Sileks, Russia), and 0.5 pMol each of forward and reverse primers (Evrogen, Russia). We used 18f (forward) primer combined with one of the reverse primers (specific to small (ss) and large (ls) subunits of rRNA): ss530r, ss1492r or ls580r to obtain fragments ~500 bp, ~1200 bp or ~1800 bp long, respectively (Weiss, Vossbrinck, 1999). The PCR conditions consisted of an initial denaturation step (95 °C for 3 min), 30 amplification cycles (denaturation at 95 °C for 30 sec; annealing at 54 °C for 30 sec, elongation at 72 °C for 30–60 sec) and a final extension step (72 °C for 10 min). The PCR products were separated in 1% agarose gels. One of the amplicons was gel purified, cloned in pAL-TA vector (Evrogen, Russia) and sequenced to confirm the specificity of the amplified band.

A new sequence was corrected manually in BioEdit and compared with Genbank entries using BLAST utility. Genbank (http://www.ncbi.nlm.nih.gov.nuccore/) was used to extract nucleotide sequence data for SSU rRNA gene of *Endoreticulatus* isolates (Table 1). The nucleotide sequences were aligned in BioEdit (Hall, 1999), nucleotide sequence similarity and pairwise genetic distances were calculated using built-in utilities of BioEdit. We used MEGA 7 software to align sequences of SSU rRNA gene of 7 isolates of genus *Endoreticulatus* from insects and *Vittaforma corneae* U11046 as the outgroup.

Phylogenetic analysis was undertaken using the Maximum Likelihood method in MEGA 7 with 500 iterations (Kumar et al., 2016). Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) was chosen using MEGA 7 built-in utility ("Find best DNA/protein models") to estimate the nucleotide substitution rate. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1799)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 42.09% sites). There were a total of 1150 positions in the final dataset.

Table 1. Genbank-accessible isolates of genus *Endoreticulatus* from insects

#	Microsporidia species/isolate	Host	Locality	Genbank accession #
1	*Endoreticulatus schubergi*	*Euproctis chrysorrhoea* (Lepidoptera: Noctuoidea)	United States	L39109
2	*Endoreticulatus bombycis*	*Bombyx mori* L. (Lepidoptera: Bombycoidea)	China	AY009115
3	*Endoreticulatus* sp. Shengzhou	*Bombyx mori* L. (Lepidoptera: Bombycoidea)	Zhejiang, China	JN688870
4	*Endoreticulatus* sp. Zhenjiang	*Bombyx mori* L. (Lepidoptera: Bombycoidea)	China	FJ772431
5	*Endoreticulatus* sp. CHW-2004 Taiwan	*Ocinara lida* Moore (Lepidoptera: Bombycoidea)	Taiwan	AY502944
6	*Endoreticulatus* sp. CHW-2004 Bulgaria	*Lymantria dispar* L. (Lepidoptera: Noctuoidea)	Bulgaria	AY502945
7	*Pleistophora* sp.*	*Spodoptera litura* Fabricius (Lepidoptera: Noctuoidea)	Japan: Tokyo, Ogasawara	LC052198
8	*Endoreticulatus* sp.	*Thaumetopoea processionea* L. (Lepidoptera: Noctuoidea)	Austria	EU260046
9	*Pleistophora* sp. Sd-N-IW8201	*Bombyx mori* L. (Lepidoptera: Bombycoidea)	Japan	D85500
10	*Pleistophora* sp. OSL-2015-2	*Spodoptera litura* Fabricius (Lepidoptera: Noctuoidea)	Japan:Tokyo, Ogasawara	LC422312
11	*Endoreticulatus poecilimonae*	*Poecilimon thoracicus* Fieber (Orthoptera: Tettigoniidae)	Bulgaria	KJ755827
12	*Endoreticulatus* sp. JMM-2007	*Loxostege sticticalis* (L.) (Lepidoptera: Pyraloidea)	Russia, Krasnodar Territory	MK929470
13	*Endoreticulatus* sp. Melnik	*Euproctis chrysorrhoea* L. (Lepidoptera: Noctuoidea)	Bulgaria	KU900486
14	*Endoreticulatus* sp. Sofia	*Euproctis chrysorrhoea* L. (Lepidoptera: Noctuoidea)	Bulgaria	KU900485
15	*Endoreticulatus* sp. WFH-2014b	*Listronotus bonariensis* Kuschel (Coleoptera: Curculionoidea)	New Zealand	KJ755828

**Pleistophora* was a collective genus name which was redefined with description of *Endoreticulatus* and other genera

Results

Examination of unfixed smears by LM revealed microsporidia spores in one moth in each of the three samples from 2006 (N=28), 2007 (N=25) and 2008 (N=36). Therefore, the prevalence of infection estimated by LM ranged from 2.8% to 4.0% (Table 2). DAPI staining confirmed the diagnosis, clearly demonstrating intensively stained double nuclei in these spores (Fig. 2). When PCR was applied for detection of microsporidia within the same samplings, positive signals (amplified band of expected size) were obtained for all LM-positive samples using each of the three primer combinations. In addition, one LM-negative sample per each of the three sampling appeared to be PCR-positive when 18f:530r (not shown) or 18f:1492r, but not 18f:ls580r primer sets, were used (Fig. 3). Thus, the prevalence rate of infection increased two-fold, ranging from 5.6% to 8% (Table 1). Average prevalence rate for the whole dataset (N=89) was 3.5±0.35% and 6.7±0.70% when estimated using LM and PCR respectively (Table 2).

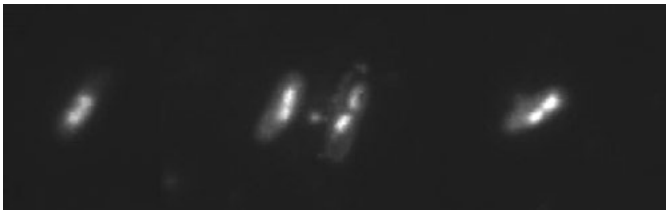

Figure 2. DAPI staining of microsporidia spores revealed in beet webworm adults from Rostov Region in 2006

Forward-end sequencing of one of the amplicons obtained using 18f:1492r primer set resulted in a ~462 bp sequence with 99.7% sequence similarity with *Endoreticulatus poecilimonae* (Genbank accession # KJ755827) and ~94% similarity with *Endoreticulatus schubergi* (# L39109) and *Endoreticulatus bombycis* (# AY009115). The majority of isolates from Lepidoptera was very similar to *E. bombycis* and *E. schubergi* (Table 3). The variable region sequenced for *E. poecilimonae* и *Endoreticulatus* sp. JMM-2007 (294 bp from 858 to 1152 position) contained 17 SNPs which were different from all other isolates but identical in these two isolates. However, similarity of these two isolates is slightly below 100% because of a single indel at position 1153 which was not reproduced in all other sequences assayed (Fig. 4) as well as in outgroup (not shown).

In the phylogram, *E. bombycis* and *E. schubergi* clustered with other isolates from Lepidoptera. *Endoreticulatus* sp. WFH 2014b from the Argentine stem weevil *Listronotus bonariensis* (# KJ755828) was in a basal position to this cluster while *E. poecilimonae* и *Endoreticulatus* sp. JMM-2007 formed a branch with 100% bootstrap support forming a sister-group relationships withthe entire aforementioned group (Fig. 5).

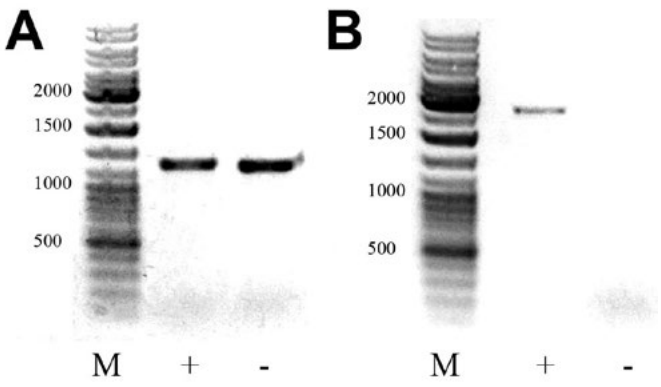

Figure 3. Electrophoretic profiles of amplification products using primers 18f:ss1492r (A) and 18f:ls580r (B) with samples of DNA, extracted from the beet webworm adults: "+" – LM-positive sample; "-" – LM-negative sample, "M" – molecular weight marker

Table 2. Microsporidia prevalence rates in beet webworm adults estimated using light microscopy and PCR

Sampling site, year	Number of analyzed insects, N	Positive samples estimated using			
		microscopy		PCR	
		n	% (n/N)	n	% (n/N)
Rostov Region, 2006	28	1	3.6	2	7.1
Krasnodar Territory, 2007	25	1	4.0	2	8
Republic of Bashkortostan, 2008	36	1	2.8	2	5.6
Total/Average(±standard error)	**89**	**3**	**3.5±0.35**	**6**	**6.7±0.70**

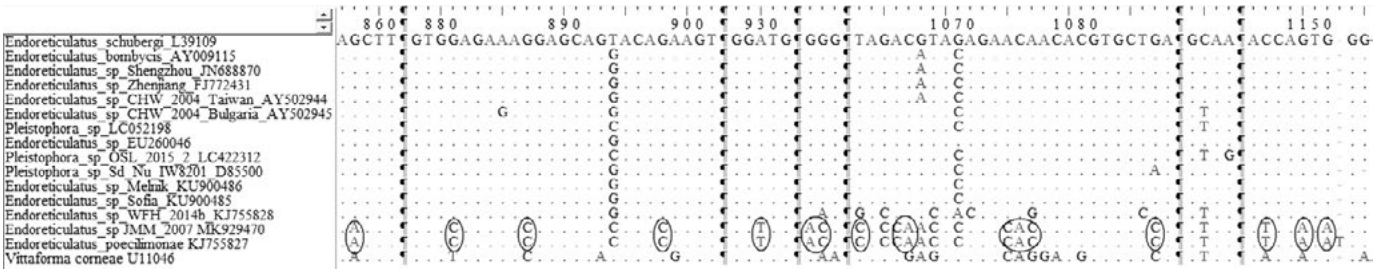

Figure 4. Alignments of nucleotide sequences of SSU rRNA gene of *Endoreticulatus* isolates. Ovals show the positions of *Endoreticulatus poecilimonae* and *Endoreticulatus* sp. JMM-2007 which are identical in these two isolates but different from the other ones

Table 3. Sequence similarity of SSU rRNA gene of *Endoreticulatus* isolates

*Isolate #	Isolate names (Genbank accession #)	Sequence similarity (lower diagonal) and genetic distance (upper diagonal)															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*Endoreticulatus schubergi* (L39109)	ID	0.011	0.008	0.008	0.008	0.013	0.008	0.005	0.011	0.005	0.005	0.014	0.041	0.064	0.064	0.137
2	*Endoreticulatus bombycis* (AY009115)	98.6	ID	0.003	0.003	0.003	0.013	0.011	0.011	0.013	0.005	0.005	0.013	0.043	0.061	0.061	0.147
3	*Endoreticulatus* sp. Shengzhou (JN688870)	98.9	99.7	ID	0.000	0.000	0.011	0.008	0.008	0.011	0.003	0.003	0.011	0.041	0.058	0.058	0.144
4	*Endoreticulatus* sp. Zhenjiang (FJ772431)	98.9	99.7	100	ID	0.000	0.011	0.008	0.008	0.011	0.003	0.003	0.011	0.041	0.058	0.058	0.144
5	*Endoreticulatus* sp. CHW 2004 Taiwan (AY502944)	98.9	99.7	100	100	ID	0.011	0.008	0.008	0.011	0.003	0.003	0.011	0.041	0.058	0.058	0.144
6	*Endoreticulatus* sp. CHW 2004 Bulgaria (AY502945)	98.4	98.6	98.9	98.9	98.9	ID	0.008	0.013	0.011	0.008	0.008	0.016	0.041	0.064	0.064	0.150
7	*Pleistophora* sp. (LC052198)	98.9	98.9	99.2	99.2	99.2	99.2	ID	0.011	0.003	0.005	0.005	0.013	0.038	0.055	0.055	0.143
8	*Endoreticulatus* sp. (EU260046)	99.2	98.9	99.2	99.2	99.2	98.6	98.9	ID	0.013	0.005	0.005	0.013	0.040	0.067	0.067	0.147
9	*Pleistophora* sp. OSL 2015-2 (LC422312)	98.6	98.6	98.9	98.9	98.9	98.9	99.7	98.6	ID	0.008	0.008	0.016	0.041	0.058	0.058	0.146
10	*Endoreticulatus* sp. Melnik (KU900486)	99.2	99.4	99.7	99.7	99.7	99.2	99.4	99.4	99.2	ID	0.000	0.008	0.038	0.061	0.061	0.147
11	*Endoreticulatus* sp. Sofia (KU900485)	99.2	99.4	99.7	99.7	99.7	99.2	99.4	99.4	99.2	100	ID	0.008	0.038	0.061	0.061	0.147
12	*Pleistophora* sp. Sd Nu IW8201 (D85500)	96.8	97.1	97.3	97.3	97.3	96.8	97.1	97.1	96.8	97.6	97.6	ID	0.041	0.068	0.068	0.149
13	*Endoreticulatus* sp. WFH 2014b (KJ755828)	95.8	95.8	96.0	96.0	96.0	96.0	96.3	96.0	96.0	96.3	96.3	94.5	ID	0.076	0.076	0.167
14	*Endoreticulatus* sp. JMM 2007 (MK929470)	93.7	94.2	94.5	94.5	94.5	93.9	94.7	93.7	94.5	94.2	94.2	92.1	92.9	ID	0.000	0.137
15	*Endoreticulatus poecilimonae* (KJ755827)	93.4	93.9	94.2	94.2	94.2	93.7	94.5	93.4	94.2	93.9	93.9	91.9	92.6	99.7	ID	0.137
16	*Vittaforma corneae* (U11046)	86.9	86.4	86.7	86.7	86.7	86.1	86.7	86.4	86.4	86.4	86.4	84.9	84.8	87.2	87.0	ID

* as given in Table 1

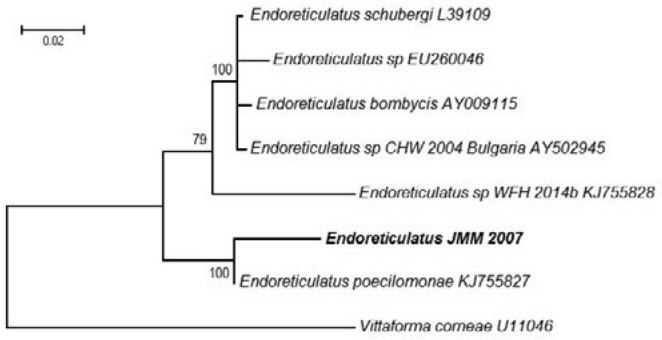

Figure 5. Molecular phylogenetic analysis by Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (see Materials and Methods)

Discussion

It has been previously shown that the sensitivity of PCR is higher for detection of microsporidia (Sokolova et al., 2004) in comparison with LM. In our study, PCR showed presence of infection in samples both containing and not containing spores. Thus, the PCR is a reliable tool for microsporidia detection even when the spores have not been formed in mass yet to be visualized by LM. Notably, comparatively long DNA fragments (~1800 bp long, amplified using 18f:ls580r in the present study) are well preserved only within the spore containing samples while shorter fragments can be amplified from samples not containing spores. Amplification of short fragments is useful for sensitive detection of the infection (Franzen, Müller, 2001) while longer fragments are required for precise species identification and elucidation of subtle genetic differences between closely related species (Tokarev, Issi, 2018). Meanwhile, microscopic examination of insect tissues remains an important part of the investigation process to estimate prevalence rate of patent infections, demonstrate spore morphology and collect spores for bioassays and other purposes.

Endoreticulatus is a genus of microsporidia infecting insects from different orders (Pilarska et al., 2015), but the majority of findings are associated with Lepidoptera. For example, *E. schubergi* was found in *Lymantria dispar, Hyphantria cunea* and *Choristoneura fumiferana* (Zwölfer, 1927; Cali, El Gary, 1991) and *E. bombycis* was described from *Bombyx mori* (Zhang et al., 1995). Several isolates of *Endoreticulatus* with unclear taxonomic position (lacking data sufficient for new species description) were reported from *Euproctis chrysorrhoea* (Pilarska et al., 2002), *Ocinara lida* (Wang et al., 2005), *Thaumetopoea processionea* (Hoch et al., 2008) and *Eilema complana* (Pilarska, 2017). All these insect hosts belong to Bombycoidea or Noctuoidea. Therefore, the finding of an isolate of *Endoreticulatus* in *L. sticticalis* extends the host range of this parasite genus to Pyraloidea.

In the first round of bioinformatics analysis performed in 2008, there were no Genbank entries showing 100% identity to the newly found rDNA haplotype. Among described species, only two (*E. schubergi* and *E. bombycis*) were genotyped and their SSU rRNA gene sequence similarity was about 94% as compared to the microsporidium found in Krasnodar Territory. These results were briefly mentioned in a presentation given at 37th Annual Meeting of the Society of Invertebrate Pathology in 2010 and in a methodological paper (Tokarev et al., 2012) and a review (Malysh et al., 2013a), both papers published locally and with some mistakes. For example, in the review paper the spore morphotype from Krasnodar Territory is incorrectly indicated as the morphotype for Novosibirsk Region (Malysh et al., 2013a, Fig. 1A) and vice versa, the morphotype from the latter sampling site is designated as the one from the former sampling site (ibidem, Fig. 2A). More recent examination of all information accumulated after that time showed identical trends in microsporidia presence and sensitivity of the two different detection methods using three independent datasets from three years of study. Moreover, high level of sequence similarity of the microsporidia from *L. sticticalis* to *E. poecilimonae* from the bellied bright bush-cricket *Poecilimon thoracicus* (# KJ755827) was also observed. Although the properly read DNA fragment was short, the sequenced region was variable among species and all other sequences showed similarity below 94% when compared to the parasites of the bellied bright bush-cricket and the beet webworm (Table 3). Based on this, we suggest that the beet webworm microsporidium belongs to the same species as *E. poecilimonae*, or at least to a group of species which are more closely related to each other than to *E. schubergi* and *E. bombycis* (Fig. 5).

Further studies are needed to elucidate the genetic borders between the *Endoreticulatus* species from different hosts and localities.

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Полнотекстовая статья

ВЫЯВЛЕНИЕ МИКРОСПОРИДИЙ, ЗАРАЖАЮЩИХ ЛУГОВОГО МОТЫЛЬКА *LOXOSTEGE STICTICALIS* (PYRALOIDEA: CRAMBIDAE) В ЕВРОПЕЙСКОЙ ЧАСТИ РОССИИ В 2006–2008 ГОДАХ

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Луговой мотылёк *Loxostege sticticalis* (L.) – опасный вредитель сельскохозяйственных культур в России, Китае и соседних странах. Микроспоридии – облигатные внутриклеточные паразиты, которые отрицательно влияют на плотность популяций многих видов насекомых, включая чешуекрылых. В частности, заражение микроспоридиями – важный фактор смертности *L. sticticalis*. Для идентификации микроспоридий наземных насекомых требуются специальные методические подходы. В настоящей работе мы описываем результаты мониторинга микроспоридий в имаго лугового мотылька с помощью двух методик – световой микроскопии и ПЦР. Имаго собирали в 2006–2008 в Европейской части России: Ростовской области, Краснодарском крае и в Республике Башкортостан. Заражение микроспоридиями выявлено в насекомых, собранных во всех точках сбора. Исследование мазков с помощью световой микроскопии показало присутствие спор микроспоридий в 3.4% образцов (N=98). ПЦР-анализ этой же выборки был положителен для 6.7% образцов, включая таковые, содержащие и не содержащие споры. Более высокие показатели зараженности, выявленные с помощью ПЦР, скорее всего, связаны с тем, что только зрелые споры могут быть однозначно идентифицированы с помощью световой микроскопии, тогда как ПЦР позволяет выявлять другие стадии развития микроспоридий. Частичное секвенирование ампликона из Краснодарского края показало его высокое родство с *Endoreticulatus poecilomonae* из *Poecilimon thoracicus* Fieber (Orthoptera: Tettigoniidae).

Ключевые слова: микроспоридии, луговой мотылёк, диагностика, флюоресцентная микроскопия, ДАФИ, ПЦР, рДНК, *Endoreticulatus*

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