ACTA
PROTOZOOLOGICA

Acta Protozool. (2018) 57: 49–52
www.ejournals.eu/Acta-Protozoologica
doi:10.4467/16890027AP.18.004.8398

Spore Dimorphism in *Nosema pyrausta* (Microsporidia, Nosematidae): from Morphological Evidence to Molecular Genetic Verification

Inna V. GRUSHEVAYA, Anastasia N. IGNATIEVA, Svetlana M. MALYSH, Igor V. SENDERSKIY, Ivan V. ZUBAREV, Anastasia G. KONONCHUK

All-Russian Institute for Plant Protection, Pushkin, Saint-Petersburg, Russia

Abstract. Microsporidia infection rate in *Ostrinia nubilalis* larvae collected in Russia in 2011–2016 ranged from 0 to 16%. Totally, among 262 examined insects, there were as many as 13 infected specimens, resulting in average prevalence of 5% over the period indicated. In all positive samples but one diplokaryotic spores 4 μm long were observed corresponding to diagnosis of *Nosema pyrausta*. Nevertheless, in one case (i.e. 0.4%) the infected larva contained monokaryotic spores about 2 μm long. After experimental infection of a substitute host *Ostrinia furnacalis* with monokaryotic spores only *Nosema*-like spores were observed in laboratory assays. Ribosomal RNA and RPB1 gene portions were 100% identical in samples of both mono- and diplokaryotic spores. This observation shows that *Nosema pyrausta* can form uninucleate spores under yet to be described conditions in nature and that molecular genetic analysis is essential for correct species identification.

Key words: microsporidia, corn borer, life cycle, molecular genetic identification

INTRODUCTION

Microsporidia are obligate intracellular parasites of animals widely occurring in natural populations of their hosts, especially arthropods (Becnel and Andreadis 1999). In Lepidoptera, natural microsporidia infections contribute to the population density dynamics of their hosts (Franz and Huger 1971, Wilson 1973, Lipa 1976, Issi 1986, Solter and Hajek 2009, van Frankenhuyzen *et al.* 2011), including an important maize pest, European corn borer *Ostrinia (Pyrausta) nubilalis* (Hill and Gary 1979, Lewis *et al.* 2006). In France, two microsporidia were described from this insect host: *Nosema (Perezia) pyrausta* (Paillot 1927) and *Thelohania ostriniae* (Lipa 1977). In the present paper, we compare the molecular genotypes of two spore morphotypes of *N. pyrausta* isolated from field populations of *O. nubilalis* from Russia.

MATERIALS AND METHODS

Diapausing larvae of *Ostrinia nubilalis* were collected in corn fields in the vicinity of settlement Botanika, Gulkevichi District, Krasnodar Territory on annual basis from 2011 to 2016 in September–October. Live insects were transferred to the laboratory and

Address for correspondence: Inna V. Grushevaya, All-Russian Institute for Plant Protection, Podbelskogo 3, Pushkin, Saint-Petersburg, Russia, 196608; Tel./Fax: +78124705110; E-mail: grushevaya_12@mail.ru

stored at +8–12°C in glass jars provided with folded paper pieces for 4 months prior to reactivation and laboratory experiments. Each month diapausing larvae were checked visually to remove cadavers which were dried and stored at room temperature. Fresh smears were prepared from perished specimens and examined in bright field for the presence of microsporidia spores with Carl Zeiss Imager M1 equipped with epifluorescence. When microsporidia spores were observed on the slides, they were fixed and stained with DAPI. For infection assay, the spores (8×10^8) were isolated from infected tissues by homogenization in water and pelleting using a centrifuge at 4000 g for 5 min and fed to second instar larvae of a continuous laboratory culture of Asian corn borer *Ostrinia furnacalis* by addition of 4×10^6 spores per larva to the artificial diet portion. After contaminated diet consumption, the infected larvae (N = 20) were routinely maintained at +20–22°C and 18:6 (light:dark) photoperiod on normal artificial diet. Control insects were from the same infection-free colony, not treated with spores. Three weeks later larvae and pupae were dissected and examined for microsporidia infection. The experiment was repeated two times with similar results using 12 and 15 insect specimens for dissection, respectively.

Samples of microsporidia spores isolated from insects collected in field and after experimental infection were subjected to DNA extraction, amplification and sequencing (Sambrook *et al.* 1989). To avoid cross-contamination between samples during molecular genetic studies, samples of different spore morphotypes were treated independently and the whole cycle of genotyping was repeated two times with identical results for each sample. Samples from 2012, 2014 and 2016 were amplified with primers 18f:1492r (Weiss and Vossbrinck 1999) flanking small subunit ribosomal RNA (SSU rRNA) gene fragment ~1200 bp long. For a more robust molecular genetic comparison, primers nvRPB1F1 (5'-CCWATGTTYCATGTYGGTTA-3') and nvRPB1R1 (5'-TAATTACAGACCTGGCACT-3') were designed targeting the RNA polymerase II largest subunit (RPB1) gene fragment ~500 bp long of *Nosema|Vairimorpha* group based upon RPB1 nucleotide sequences of two type species: *Nosema bombycis* (# DQ996231) and *Vairimorpha necatrix* (# AF060234).

RESULTS AND DISCUSSION

Approximately 200 to 300 larvae of *O. nubilalis* were collected each year and mortality was approximately 10 to 20% during overwintering, prior to experimental activation in April–May, under laboratory conditions. Number of larvae examined using light microscopy for microsporidia infection ranged from 20 to 115, microsporidia prevalence rate ranged from 0 to 16% with infections seen each year but 2015 (number of examined insects N = 115), overall average = 5%, N = 262 (Table 1). This was consistent with previously obtained data (Malysh *et al*. 2011) for the period of 2005–2010 where percent infection was reported to range from 3 to 17%. In most cases, typical oval diplokaryotic *Nosema*-like spores were observed, measuring 4–5 μm × 2–3 μm, live (unfixed). The only exception was in 2016 where 1 of 3 infected specimens was found to harbor small (about 2 μm × 1.5 μm in size, unfixed) monokaryotic spores (Fig. 1). The overall average of monokaryotic spores is therefore 0.4% (N = 262). The cadaver containing these spores was partially liquefied, not suitable for detection of sporophorous vesicles in these spores (characteristic of an additional octosporous sporogony in Nosema/Vairimorpha) or for ultrastructural analysis. In an experimental infection assay, feeding these monokaryotic spores to infection-free *O. furnacalis* larvae resulted in the formation of diplokaryotic spores 4–5 μm long in 100% of examined insects (N = 27, pooled from two experiments).

All SSU rRNA gene sequences of microsporidia isolated from *Ostrinia* in this paper were 100% identical to the type sequence reported for the European isolates of *Nosema pyrausta* (Genbank accession # HM566196) discovered both in France and Krasnodar Territory (Tokarev *et al.* 2015) and 99.8% similar to *Nosema bombycis* (Table 2). RPB1 nucleotide sequence (# MG182018) was 100% identical in microsporidia with diplo- and monokaryotic spores from *O. nubila-*

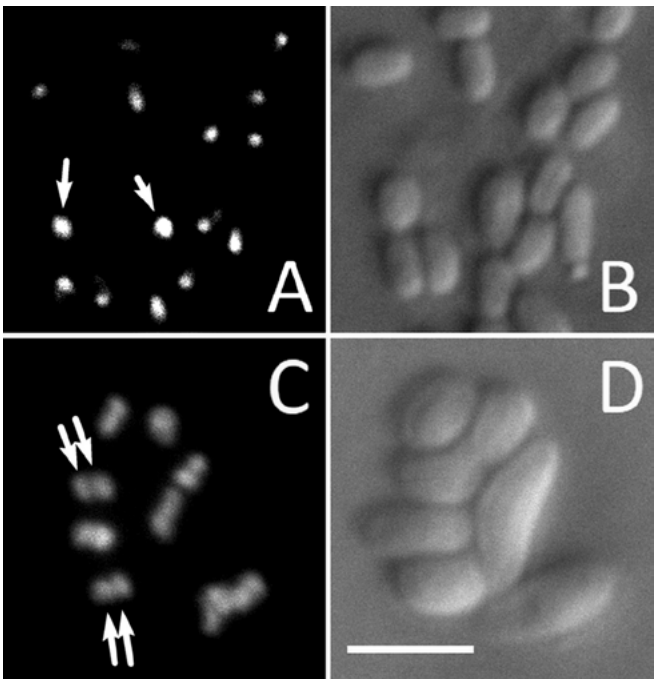

Fig. 1. DAPI fluorescence (A, C) and Nomarski contrast (B, D) of monokaryotic (A, B) and diplokaryotic (C, D) spores of microsporidia detected in *Ostrinia nubilalis* larvae. Arrows and double arrows indicate single nuclei and diplokarya, respectively. Scale bar = 4 μm.

lis. When compared to Genbank, it showed about 90% identity to *N. bombycis* (Table 3).

This is another example of an additional sporogony observed in true *Nosema,* others being «*Vairimorpha*» imperfecta, «*Vairimorpha*» *cheracis* and *Nosema distriae* (Kyei-Poku and Sokolova 2017). The incidence rate of the monokaryotic spore morphotype is about tenfold as lower as compared to the diplokaryotic one, which is consistent with observations in other members of the *Nosema/Vairimorpha* group. Interestingly, *Thelohania*-like spores were occasionally found in an *O. nubilalis* population (Lipa 1977). Based upon this morphological character and revealing this spore morphotype in a novel host, this microsporidium was described as a new species, *Thelohania ostriniae.* Nevertheless, taking into consideration that the very population was

Table 1. Percentage of infection with microsporidia in *Ostrinia nubilalis* larvae collected in field and perished in lab during hibernation

Year of collection	Cadavers with microsporidia spores		Morphotype	SSU rRNA gene identity
	n(N)	% ± SE		
2011	1(20)	4.0 ± 3.9	*Nosema*-like	NA
2012	4(25)	16 ± 7.3	*Nosema*-like	*N. pyrausta*
2013	2(33)	6.7 ± 4.6	*Nosema*-like	NA
2014	3(30)	10 ± 5.5	*Nosema*-like	*N. pyrausta*
2015	0(115)	0	–	NA
2016	3(25)	8.1 ± 4.5	*Nosema*-like (2); *Thelohania*-like (1)	*N. pyrausta*
Total	13(262)	5.0 ± 1.3	–	–

n(N) – total number of examined (N) and infected insects (n); NA – not assayed; SE – standard error

Table 2. Sequence similarity of SSU rRNA gene portion sequenced for isolates of *Nosema pyrausta* and *Nosema bombycis*

#	Species	Host	Genbank accession #	Sequence similarity, %			
				1	2	3	4
1	*Nosema bombycis*	*Bombyx mori*	AY209011	=	–	–	–
2	***Nosema pyrausta***	*Ostrinia nubilalis*	HM566196	99,8	=	–	–
3	*Nosema pyrausta*	*Ostrinia nubilalis**	ND	99,8	100	=	–
4	*Nosema pyrausta*	*Ostrinia furnacalis***	ND	99,8	100	100	=

* monokaryotic spores from a field collected sample
** diplokaryotic spores from a substitute host infected artificially under lab conditions
ND – not deposited
The type isolate of *N. pyrausta* is in bold

Table 3. Sequence similarity of RPB1 gene portion sequenced for isolates of *Nosema pyrausta* and *Nosema tyriae* in the present study (in bold) and related sequences available from public databases

#	Species	Host	Genbank accession #	Sequence similarity, %			
				1	2	3	4
1	*Nosema bombycis*	*Bombyx mori*	DQ996231	=	–	–	–
2	***Nosema pyrausta***	*Ostrinia nubilalis*	MG182018	92,0	=	–	–
3	*Nosema pyrausta*	*Ostrinia nubilalis**	ND	92,0	100	=	–
4	*Nosema pyrausta*	*Ostrinia furnacalis***	ND	92,0	100	100	=

All indications as in Table 2

also co-infected with *N. pyrausta*, it is also possible that there also was an additional sporogony of the latter species producing *Thelohania*-like spores, as assumed in the present study. Unfortunately, this assumption cannot be validated. It also cannot be concluded, whether specific conditions are needed for *Thelohania*-like spore production in *N. pyrausta* or this process is sporadic. The biological importance of an additional *Thelohania*-like sporogony in the life cycle of *N. pyrausta* is obscure; some authors link this developmental sequence with sexual process and consider these spores as meiospores i.e. resulted from meiosis (Kyei-Poku, Sokolova 2017).

These observations indicate that (a) two morphotypes of microsporidia spores found in corn borer larvae under field conditions belong to the same species of the parasite, namely *N. pyrausta*; (b) the monokaryotic spores of *N. pyrausta* are met at frequency below 0.5% in Krasnodar Territory; (c) monokaryotic spores are infective to corn borer larvae but under lab conditions in a substitute host this morphotype is switched to a regular developmental sequence resulting in diplokaryotic spores; (d) RPB1 gene sequencing is exploitable for differentiation of closely related species and geographic isolates of microsporidia.

Acknowledgements. The authors are thankful to Yuri S. Tokarev for advice. The research is supported by Russian Science Foundation, project # 16-14-00005. Authors have no competing interests to declare.

REFERENCES

Becnel J. J., Andreadis T. G. (1999) Microsporidia in insects. In: Wittner M., Weiss L. M., (eds.), *The Microsporidia and microsporidiosis*, American Society Microbiology Press, Washington DC. 447–501

Franz J. M., Huger A. M. (1971) Microsporidia causing the collapse of an outbreak of the green tortrix *Tortrix viridana* L. in Germany. *Proc. Internat. Colloq. Insect Pathol. 4th College Park*, MD. 48–53

Hill R. E., Gary W. J. (1979) Effects of the microsporidium, *Nosema pyrausta*, on field populations of European corn borers in Nebraska. *Environ. Entomol.* **8:** 91–95

Issi I. V. (1986) Microsporidia as a phylum of parasitic protozoa. *Protozoologiya.* (Leningrad) **10:** 1–136 (in Russian)

Kyei-Poku G., Sokolova Y. Y. (2017) The microsporidium *Nosema disstriae* (Thomson 1959): Fine structure and phylogenetic position within the *N. bombycis* clade. *J. Invertebr. Pathol.* **143:** 90–103; https://doi.org/10.1016/j.jip.2016.12.003

Lewis L. C., Sumerford D. V., Bing L. A., Gunnarson R. D. (2006) Dynamics of *Nosema pyrausta* in natural populations of the European corn borer, *Ostrinia nubilalis*: A six-year study. *Biocont.* **51:** 627–642

Lipa J. J. (1976) Microsporidians parasitizing the green tortrix in Poland and their role in the collapse of the tortrix outbreak in Puszcza Niepolomicka during 1970–1974. *Acta Protozool.* **15:** 529–536

Lipa J. J. (1977) *Thelohania ostriniae* n.sp., a new microsporidian parasite of the European corn borer *Ostrinia nubilalis* Hnb. (Lepidoptera, Pyralidae). *Acta Protozool.* **16:** 151–155

Malysh Yu. M., Tokarev Yu. S., Sitnikova N. V., Kononchuk A. G., Gruschetskaya T. A., Frolov A. N. (2011) Incidence of microsporidian infection of stem borers of the genus *Ostrinia* (Lepidoptera: Crambidae) in the Krasnodar Territory. *Parazitologiya.* **45:** 234–243 (in Russian)

Paillot A. (1927) Sur deux protozoaires nouveaux parasites des chenilles de *Pyrausta nubilalis* Hubner. *Compt. Rend. l'Acad. Sci. Paris* **185:** 673–675

Sambrook J., Fritsch E., Maniatis T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Solter L. F., Hajek A. E. (2009) Control of gypsy moth, *Lymantria dispar*, in North America since 1878. Use of microbes for control and eradication of invasive arthropods. Springer Netherlands. 181–212

Tokarev Yu. S., Malysh J. M., Kononchuk A. G., Seliverstova E. V., Frolov A. N., Issi I. V. (2015) Redefinition of *Nosema pyrausta* (*Perezia pyraustae* Paillot 1927) based upon ultrastructural and molecular phylogenetic studies. *Parasitol. Res.* **114:** 759–761; https://doi.org/10.1007/s00436-014-4272-3

van Frankenhuyzen K., Ryall K., Liu Y., Meating J., Bolan P., Scarr T. (2011) Prevalence of *Nosema* sp. (Microsporidia: Nosematidae) during an outbreak of the jack pine budworm in Ontario. *J. Invertebr. Pathol.* **108:** 201–208; https://doi.org/10.1016/j.jip.2011.09.002

Weiss L. M., Vossbrinck C. R. (1999) Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the Microsporidia. In: Wittner M., Weiss L. M. (eds.), *The Microsporidia and microsporidiosis*. American Society Microbiology Press, Washington DC. 129–171

Wilson G. G. (1973) Incidence of microsporidia in a field population of a spruce budworm. *Bimonth. Res. Can. For Serv.* **29:** 35–60

Received on 7th December, 2017; revised on 27th January, 2018; accepted on 18th February, 2018