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Short Communication

Identification of *Nosema bombi* Fantham and Porter 1914 (Microsporidia) in *Bombus impatiens* and *Bombus sandersoni* from Great Smoky Mountains National Park (USA)

Yuliya Y. Sokolova [a,b,c,*], Igor M. Sokolov [b,d], Christopher E. Carlton [b]

[a] Department of Entomology, Louisiana State University AgCenter, 404 Life Sciences Bldg., Baton Rouge, LA 70803-1710, USA
[b] Institute of Cytology, Russian Academy of Sciences, 4 Tikhoretsky Ave., 194064 St. Petersburg, Russia
[c] Tulane National Primate Research Center, Div. Microbiology, 18703 Three Rivers Road Covington, LA 70433-8915, USA
[d] Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, 44 M.Thorez Prospect, 194223 St. Petersburg, Russia

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ABSTRACT

Ninety three bumble bees belonging to the genus *Bombus*, subgenus *Pyrobombus* (three *Bombus vagans*, seven *Bombus bimaculatus*, 17 *B. sandersoni* and 68 *B. impatiens*) from Great Smoky Mountains National Park were examined for microsporidia. Light microscopy of calcofluor and trichrome-stained smears, and PCR revealed infection with *N. bombi* in one specimen each of *B. sandersoni* and *B. impatiens*. Sizes and shapes of spores in both *N. bombi* isolates were similar to those described for European isolates of the microsporidium. A region of the rRNA gene from the *B. impatiens* isolate (1689 bp, accession GQ254295) aligned with homologous sequences from eight European isolates, with only three variable sites. Sequence variability of this region between novel isolates and the European ones was the same as among European isolates.

1. Introduction

Infection with the microsporidium *Nosema bombi* has been suggested as a potential causes of the decline of bumble bees during last two decades reported in several regions of the USA (Grixti et al., 2009; Plischuk et al., 2009), including Great Smoky National Park (GSMNP) (A. Mayor, Curator, GSMNP, personal communication). In Europe, natural infections with *N. bombi* have been recorded in 10 *Bombus* spp. (Larsson, 2007; Tay et al., 2005) belonging to five subgenera (Cameron et al., 2007). Morphological and genetic variations of European *N. bombi* isolates infecting different *Bombus* species have been described (Larsson, 2007; Tay et al., 2005). In the United States, infection with *N. bombi* has been documented for *Bombus occidentalis* imported from Canada for laboratory experiments (Whittington and Winston, 2003). This is the only documented record of this microsporidium occurring in the US except for an unconfirmed case in *B. occidentalis* colonies grown commercially in California in 1997 (Thorp, 2003). Previous authors speculated that the microsporidium probably was released in the USA during 1992 via release of native *B. occidentalis* and *Bombus impatiens* commercially reared in European facilities (Thorp, 2003), though no evidence or references were provided. No *N. bombi* isolates from *Bombus* spp., native to the USA, have been characterized until the present study.

Do native bumble bees that inhabit GSMNP, harbor *N. bombi*? What is the host range and prevalence of infection, and does it create a threat to bumble bee populations native to the USA? To address those questions we undertook a pilot survey for microsporidia among *Bombus* spp. in GSMNP.

2. Material and methods

All insects were collected in GSMNP remote from sources of reared bumble bees. A total of 93 bumble bees belonging to the genus *Bombus*, subgenus *Pyrobombus*, were examined for microsporidian infection. Among those were: three *Bombus vagans*, seven *Bombus bimaculatus*, and 17 *Bombus sandersoni*, all stored in 90% ethyl alcohol at room temperature since 2002–2003. These specimens were provided by A. Mayor, Curator, GSMNP. In addition, 68 *B. impatiens* were collected by hand by the first and second authors for this study during July 26, 2008 and stored in 96% ethyl alcohol at −20 °C. Smears from abdomens were fixed with methanol, stained with Trichrome blue and Calcofluor stains (Remel, Lenexa, KS), and viewed under a Leica DM LM microscope at 400–1000×. Spores were photographed and measured using a SpotInsight digital camera and implemented software.

For DNA extractions soft tissues were isolated from abdomens and transferred to 150 µl of lysis guanidine buffer (4.2 M guanidine

* Corresponding author. Address: 4 Tikhoretsky Ave., Institute of Cytology, Russian Academy of Sciences, 194064 St. Petersburg, Russia. Fax: +7 812 297 0341.
E-mail address: jumicro@yahoo.com (Y.Y. Sokolova).

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thyocyanate, 50 mM Tris–HCl, pH 7.6, 10 mM EDTA, 25% lauryl sarcosinate + 10% mercaptoethanol), bead-beaten in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) at maximum speed for 30 s, and heated in a thermoblock for 10 min at 95 °C. Afterwards the samples were spun down, and the supernatants were subjected to phenol–chloroform–isoamyl alcohol extraction followed by alcohol precipitation. The DNA pellet was dissolve in 40 μl of deionized water and used as a DNA template in PCR reactions. PCR was performed as described in Klee et al. (2006) with SSUrRNA-f1/r1c, "universal" (working with *N. bombi, N. apis*, and *N. ceranae*), and "specific" *N. bombi*-SSU-Jf1/Jr1, primers (Klee et al., 2006). SSUr RNA, ITS and beginning of LSUr RNA genes were amplified and sequenced as in Tay et al. (2005) using SSUrRNA-f1 – SSUrRNA-r1b, SSUrRNA-f2 – SSUrRNA-r2, ITS-f1 – ITS-r1, and ITS-f2 – ITS-r2 primer pairs (Tay et al., 2005). The sequencing reactions were performed with Applied BioSystems BigDie Terminator (version 3.1) and run on the Beckman Coulter Seq 8000 DNA sequencer in the GenLab, School of Veterinary Medicine, Louisiana State University. The primers produced overlapping sequences that were assembled with Chromas. Pro. 1.34 software (http://www.technelysium.com.au/ChromasPro.html). The obtained contigs were aligned with the homologous sequences available from Genebank (AY008373 and AY74101–AY74111) with the CLUSTAL X program (Thompson et al., 1997).

3. Results and discussion

Three detection methods used (two stains and PCR) were consistent and revealed microsporidia infection only in two of 93 examined bumble bees, one each of *B. sandersoni* and *B. impatiens*. Specimen data from the two infected insects were: *B. sandersoni*, NC Swain Co PT56 04.14-05.19 2003; *B. impatiens*, TN Blount Co Abrams Creek Ranger St. 07-26-2008. Spores, found in both host species, varied in shape from elongated to oval. Spores from *B. sandersoni* ranged from 2.7–4.5 × 1.4–2.3 μm and averaged 3.6 ± 0.08 × 1.9 ± 0.04 μm (n = 37; x ± SE). Spores from *B. impatiens* ranged from 2.5–5.2 × 1.7–2.4 μm and averaged 3.3 ± 0.15 × 1.93 ± 0.06 μm (n = 24) (Fig. 1a–c). Spore size and morphology as well as variability of these parameters were similar to those recorded for *N. bombi* isolates from European bumble bees (Fries et al., 2001; Larsson, 2007).

PCR amplification of DNA isolated from 93 bumblebees with SSUrRNA-f1-r1c (universal), and *N. bombi*-SSU-Jf1/Jr1 (specific) pairs of primers confirmed the diagnosis. Amplicons were produced only in the two DNA samples that were isolated from bumble bees positive for microsporidia in light microscopy. Sequences of the amplicons produced by *N. bombi*-SSU-Jf1/Jr1 were 323 and 321 bp for DNA *B. sandersoni* and *B. impatiens* isolates, respectively. They were identical and exactly matched the correspondent regions (positions 1–323 of the ribosomal gene (Klee et al., 2006; Tay et al., 2005) of the European isolates of *N. bombi*. The amplification of a larger region of the ribosomal gene produced 1207 bp fragment in *B. sandersoni* and 1689 bp fragment in *B. impatiens*. The latter fragment was deposited in Genebank under accession no: GQ254295. The 1207 fragment (positions 1–1207) were identical for both isolates and matched exactly the AY008373 sequence (*Bombus terrestris* isolate of *N. bombi* (Fries et al., 2001)) and the "consensus" sequence (consensus from AY741101 to AY741109 (Tay et al., 2005)), except for position 189, which in our sequences was "A" instead of "R". The ITS region (positions 1261–1268) of the *B. impatiens* isolate contained two repeats of GTTT motifs. Only three variable sites at positions 1534, 1537, and 1540 were revealed when the 1689 bp SSUrDNA fragment of this *N. bombi* isolate was aligned with corresponding regions of the isolates infecting eight European *Bombus* spp. (Fig. 2).

Our pilot study shows that *N. bombi* does infect bumble bees native to the U.S., indicating that the geographical distribution and host range of this microsporidium is broader than previously reported. *B. sandersoni* and *B. impatiens* were represented in our study by greater numbers of specimens than *B. vagans*, and *B. bimaculatus*, which was probably the only reason infections were discovered in the former two species, but not the latter. The incidence of *N. bombi* infection in *B. sandersoni* and *B. impatiens* was similar to that reported for some species of European bumble bees (Larsson, 2007). More extensive sampling is necessary to estimate real occurrence of the microsporidium among bumble bee populations in GSMNP, however our data suggest no signs of an epizootic or elevated level of infections, at least in *B. impatiens*. But other species (e.g., *Bombus affinis, Bombus terricola*, and *Bombus pensyvanicus*) were not analyzed, and we cannot argue the role of microsporidiosis in rapid declines of these species during the last decade. In spite of geographically distant areas of distribution and parasitism of different *Bombus* spp., the SSUrDNA sequence divergence between the two *N. bombi* isolates described here and the European isolates was minimal, and not greater than among European isolates themselves. Microsporidia parasitism in insects is phylogenetically ancient, GSMNP is geographically remote from California, and *B. impatiens* and *B. sandersoni* belong to two distant lineages of the Holarctic subgenus *Pyrobombus* (Cameron et al., 2007; Kawakita et al., 2004). We therefore presume that *N. bombi* was present in ancestors of contemporary Nearctic *Bombus* spp. and arrived with their hosts following multiple dispersals from the Palearctic (Cameron et al., 2007; Kawakita et al., 2004). This seems more likely than a recent origin via spread from non indigenous infections such as those mentioned previously. A hypothesis that colonization of US bumble bee populations with *N. bombi* occurred recently cannot be rejected, but appears highly unlikely, based on available data. Further sampling of various *Bombus* spp. throughout North America and analyzes of other than rRNA genes,

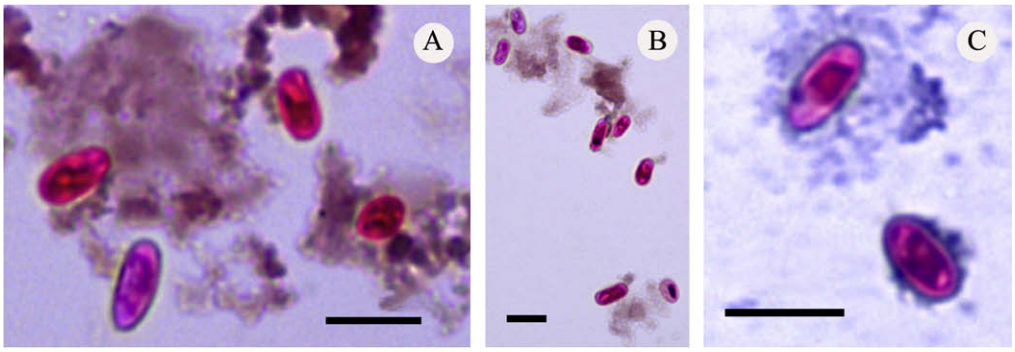

Fig. 1. Spores of *Nosema bombi* in smears of abdomens of *Bombus sandersoni* (A and B) and *B. impatiens* (C) stained with Trichrome Blue. Magnification: A and C, 100×; B, 40×. Bar = 5 μm.

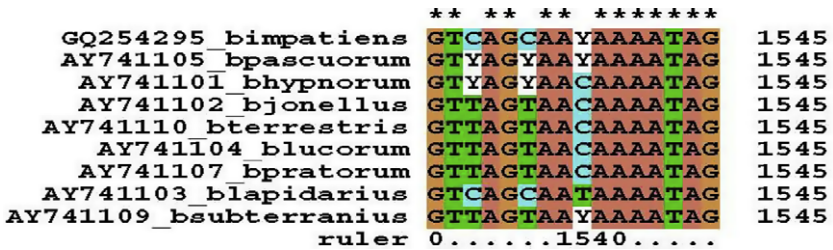

Fig. 2. Fragment of the alignment of 1689 bp SSUrDNA fragment of *N. bombi* isolated from *B. impatiens*, against homologous regions of isolates infecting eight European *Bombus* spp. Only three variable sites at the positions 1534, 1537 and 1540 were revealed.

such as RNA polymerase II (Ironside, 2007; Shafer et al., 2009), might help trace dispersal patterns of this parasite.

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