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Pathogenicity, morphology, and characterization of a *Nosema fumiferanae* isolate (Microsporidia: Nosematidae) from the light brown apple moth, *Epiphyas postvittana* (Lepidoptera: Tortricidae) in California

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ABSTRACT

We recently discovered infections by a microsporidium closely related to *Nosema fumiferanae* in field populations of the light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), in the San Francisco region of California. *E. postvittana* originates from Australia and was first detected in California in 2006; therefore, our aim was to identify and determine the origin of the *Nosema* isolate. We characterized the pathogenicity, transmission pathways, and ultrastructure of this new *Nosema* isolate. In addition, we sequenced fragments of commonly used genetic markers (ITS, SSU, and RPB1), and examined the phylogenetic relationships between the *Nosema* isolate and other microsporidian species commonly found in lepidopteran hosts. The pathogenicity of the *Nosema* isolate was investigated by infecting second instar larvae of *E. postvittana*. Larval and pupal survivorship were reduced by 7% and 13% respectively, and pupation occurred 1–2 d later in infected individuals than in healthy individuals. Emerging infected females died 5 d earlier than healthy females, and daily fecundity was 22% lower. Hatch rate also was 22% lower for eggs oviposited by infected females. Vertical transmission was confirmed; spores were present in 68% of egg masses and 100% of the surviving larvae from infected females. Ultrastructure images, together with sequences from selected genetic markers, confirmed the *Nosema* isolate to be a member of the *Nosema fumiferanae* species complex (*Nosema fumiferanae postvittana* subsp. n.). The association of this pathogen with *E. postvittana* contributes further to the biotic resistance that *E. postvittana* has experienced since its introduction to California.

1. Introduction

Microsporidia are a diverse group of intracellular pathogens suggested to belong to a newly erected superphylum, Opisthosporidia, a deep-branch clade of Holomycota related to the Fungi (Karpov et al., 2014; Keeling, 2014) and comprise over 185 described genera and over 1300 species that infect protists, invertebrates and vertebrates, including humans (Solter et al., 2012a; Vavra and Lukes, 2013). There are more than 150 described species in the genus *Nosema* (Microsporidia: Nosematidae) that are associated with 12 different orders of insects (Becnel and Andreadis, 2014). The type species of the genus, *Nosema bombycis* (Nägeli) was the first member of the Microsporidia to be described, and is the causal agent of pébrine disease in the silkworm, *Bombyx mori* L. (Vavra and Lukes, 2013; Becnel and Andreadis, 2014). In addition to the impact of *N. bombycis* in sericulture, several microsporidian species have expanded their geographic range either as accidental or deliberate introductions. *N. ceranae* Fries, suggested to originate from *Apis cerana* F. in Asia (Fries et al., 1996; Gomez-Moracho et al., 2015), has been found in Europe, North America, and South America where it infects *A. mellifera* L. and bumble bees (Graystock et al., 2013). *N. tyriae* Canning was accidentally introduced from Europe to North America along with the deliberate introduction of its host, the cinnabar moth, *Tyria jacobaeae* (L.) (Hawkes, 1973), and *N. lymantriae* Weiser and *Vairimorpha disparis* (Timofejeva) were deliberately introduced from Europe to North America in trial studies for biological control of the gypsy moth, *Lymantria dispar* (L.) (Hajek and Delalibera, 2010).

In 2013, we discovered an infection by a microsporidium resembling a *Nosema* species in a laboratory colony and in field populations of the light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), in the San Francisco region of California. *E. postvittana* is an exotic leaf-roller native to Australia,

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with a host plant range that includes over 500 plant species (Suckling and Brockerhoff, 2010), and was originally detected in California in 2006 (Brown, 2007). Since its introduction, *E. postvittana* has been effectively colonized by a range of resident insect parasitoids, some of which are contributing significant biotic resistance to the spread of this new invader (Wang et al., 2012; Bürgi and Mills, 2014; Bürgi et al., 2015). Thus, it was of interest to characterize this microsporidium and determine its pathology in *E. postvittana*. Initial sequence information indicated that this new isolate resembled *Nosema fumiferanae* (Thompson).

N. fumiferanae is a well-characterized microsporidium of the Eastern spruce budworm *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae). *C. fumiferana* is one of the most destructive forest defoliators in North America with its common host plants including balsam fir, *Abies balsamea* (L.) and white spruce, *Picea glauca* (Moench) Voss (Zhao et al., 2014). During an outbreak, up to 80% of *C. fumiferana* can be infected with *N. fumiferanae* (Thomson, 1960), but more generally it is a low virulence pathogen with a prevalence that varies between 10% and 50% (Eveleigh et al., 2012). Transmission of *N. fumiferanae* occurs horizontally through consumption of spores (Campbell et al., 2007) and vertically from the parent female to her offspring (Thomson, 1957; Bauer and Nordin, 1989; van Frankenhuyzen et al., 2007). The midgut of the host is the principal site of infection, and the spores eventually spread to the Malpighian tubules, fat body, silk glands, epidermis, gonads, hind gut and nerve tissue (Thomson, 1955). Infection can result in lethal or sublethal effects depending on spore load and age of the host, with younger larvae being more susceptible to lethal effects than older larvae (Thomson, 1955). Larvae of *C. fumiferana* with sublethal *N. fumiferanae* infections have prolonged development to pupation, and reduced pupal weight, fecundity and adult longevity (Thomson, 1957). In the laboratory, spores of *N. fumiferanae* have also been found in the midgut lumen of two parasitoids of *C. fumiferana*: *Apanteles fumiferanae* Vier. and *Glypta fumiferanae* (Vier.) (Thomson, 1958). In addition, laboratory tests have shown that *N. fumiferanae* can have pathogenic effects in other lepidopteran hosts, including the oblique-banded leafroller, *Choristoneura rosaceana* (Harris) (Cossentine and Gardiner, 1991) and the western tent caterpillar, *Malacosoma californicum pluviale* (Dyar) (Wilson, 1974). However, currently *N. fumiferanae* has only been confirmed from field populations of *C. fumiferana*.

Historically, identification of *N. fumiferanae* has been based on host association, pathological and morphological characteristics such as the primary site of infection, spore size, type of cell division, number of nuclei per cell, and ultrastructural details (Thomson, 1955; Percy, 1973). However, detailed morphological descriptions of microsporidia remain limited as they require ultrastructural images using transmission electron microscopy (TEM) (Solter et al., 2012b). As *Nosema* species often exhibit limited pathological and morphological differences, ribosomal DNA (rDNA) sequences recently have been used for more accurate identification (Solter et al., 2012b). Although a draft genome of *N. bombycis* and additional proteomic data are available for optimization of molecular markers (Pan et al., 2013), only rDNA loci have consistently been used for differentiating microsporidian species. These loci include the internal transcribed spacer (ITS) and the small subunit (SSU) rRNA gene, which can be used to distinguish the 'true' *Nosema* group from other microsporidia (Huang et al., 2004; Solter et al., 2012b). An additional potentially useful single copy nuclear marker is RNA polymerase II subunit (RPB1) (Cheney et al., 2001). This locus has proved useful in supporting higher level relationships, e.g. between microsporidia and fungi (Hirt et al., 1999), as well as for identification of several microsporidian genera and species (Cheney et al., 2001; Vavra et al., 2006a; Gisder and Genersch, 2013; Luo et al., 2014).

Here, we characterize the pathogenicity, transmission pathways, and morphology of a *Nosema* isolate from *E. postvittana*, *Nosema fumiferanae postivittana* subsp. n. (hereafter *N. fumiferanae postvittana*). We also sequenced three commonly used genetic markers (ITS, SSU, and RPB1) to identify the isolate and compare it to other *Nosema* species from lepidopteran hosts. We include further TEM imaging of *Nosema fumiferanae* from *C. fumiferana* (hereafter *N. fumiferanae*); previous ultrastructural images did not include the mature spore stage (Percy, 1973). We compare the ultrastructure of the two *N. fumiferanae* isolates, and relate the sequence and ultrastructure characteristics to other microsporidia in the *N. bombycis* group.

2. Materials and methods

2.1. Microsporidia isolates

Microsporidian spores were isolated in 2013 from a laboratory colony of *E. postvittana* that was initially established from larvae collected in Santa Cruz, California in 2007 and supplemented with adult females collected from Richmond and Berkeley, California in 2013. Spores of *N. fumiferanae* were originally collected from *C. fumiferana* in Sault Ste. Marie, Ontario, Canada in 2001 and stored in liquid nitrogen at −80 °C prior to use.

2.2. Host colonies and spore production

Infected and healthy host colonies were maintained at 20 °C, a 16:8 h L:D photoperiod, and a relative humidity above 60%, and all experiments were conducted under the same conditions. The healthy colony was located in an insectary and separate growth chambers were used for the infected colony and for all experiments. We used 10% bleach to sterilize all tools, containers and surfaces prior to use. An uninfected (healthy) laboratory colony of *E. postvittana* was established from egg masses donated by USDA–APHIS from a separate colony of the same original source population from Santa Cruz, California. Larvae were reared on an artificial bean-based diet developed by Cunningham (2007). Upon pupation, an equal number of pupae of each sex were transferred to 956 ml ventilated plastic oviposition cups. Prior to adult emergence, the cups were provided with 10% honey-water with 0.1% sorbic acid via a 4 cm cotton wick in a 22 ml plastic cup. Following oviposition, freshly laid egg masses were sterilized following Singh et al. (1985) in a 5% formaldehyde solution for 20 min, soaked in water for 20 min, and air dried in a sterile area before being transferred to 96-ml plastic cups containing approximately 30 ml artificial diet. We routinely evaluated larvae from the healthy colony to ensure that no microsporidian infections had occurred in the healthy insects.

An infected colony of *E. postvittana* was reared as above with the addition of 10^5 *N. fumiferanae postvittana* spores per ml mixed into the artificial diet. We utilized infected ultimate instar larvae to obtain pure spore suspensions of the isolate by homogenizing the larvae in DI water with a plastic pestle and filtering the homogenate through nylon mesh to remove host tissues and integument. We followed the 'triangulation method' of purification by Cole (1970) to further purify the homogenate. We used a hemocytometer and phase contrast microscopy (400×magnification) to count the number of spores per ml from individual larvae taken from the infected colony. We subsequently diluted the purified spore homogenate with DI water to obtain a concentration of 5×10^2 spores per μl.

2.3. Pathology and vertical transmission

To determine the effects of *N. fumiferanae postvittana* infection in *E. postvittana*, experimental second instar larvae in 22-ml plastic