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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

cups were starved for 24 h prior to providing a 2-mm cube of artificial diet with either 2 μl distilled water (healthy treatment) or 2 μl spore homogenate of *N. fumiferanae postvittana* (infected treatment). After 24 h when the 2-mm cube of diet had been completely consumed, we provided all larvae a fresh 1.5-cm cube of diet (no inoculum), and refreshed the diet cube every week. We recorded larval and pupal development time and survivorship, sex ratio (percent female) at pupation, pupal fresh weight, adult emergence, proportion of females that oviposited, per capita daily fecundity, and egg hatch. Pupae were weighed 24–48 h after pupation. Freshly eclosed females were placed with two healthy males in an oviposition cup and the date of female death was recorded. We collected all egg masses and stored them individually in sterile cups with moist cotton wicks to record both the number and the proportion of egg hatch. To confirm that healthy individuals remained uninfected, we monitored larval frass for the presence of spores from healthy and infected individuals at the time of pupation (or earlier if larval mortality occurred) by examining smears in 2 μl water on glass slides under phase contrast microscopy, 400×magnification.

We infected 57 second instar larvae with 10^3 *Nosema* spores as described above, and subsequently monitored their development until 22 surviving female moths emerged. These females were mated with healthy males and oviposition was monitored as described above. Following initial oviposition (day 1), each female was transferred to new oviposition cups on days 1, 5, and 9 to allow the collection of fresh uncontaminated egg masses on days 2, 6 and 10 of oviposition. The females were returned to their original oviposition cups on days when egg masses were not collected for evaluation. The number of eggs per egg mass were counted prior to microscopic examination to account for any correlation between egg mass size and spore presence and infection intensity. Half of the egg masses collected on days 2, 6, and 10 were examined immediately, and the other half were allowed to hatch. Five larvae from each egg mass were examined. Egg masses and larvae were smeared onto glass microscope slides in 2 μl of water. To determine whether spores were present, observations were made at 20 random fields of view at 400× magnification.

2.4. Tissues infected and morphology

To determine the progression of infection and occurrence of spores in different tissues of *E. postvittana*, we infected larvae just prior to molting to fifth instar with 10^3 spores of both microsporidian isolates and maintained them on artificial diet as described above. After molting to fifth instar, we dissected five larvae infected with the *N. fumiferanae postvittana* isolate every 24 h and rinsed freshly dissected tissues in 0.9% saline solution before smearing them on glass microscope slides under glass coverslips. We examined the tissues using 20 random fields of view under phase contrast microscopy at 400×magnification. We confirmed spore presence when necessary by staining the tissues with a modified Giemsa stain (Sigma–Aldrich) (Becnel, 2012) and examining the samples at 1000× magnification. We measured the length and breadth of 10 mature spores of both isolates with ImageJ® using an ocular scale at 1000× magnification. Measurements did not include the refractory spore border.

To compare the morphology of the two *Nosema* isolates with transmission electron microscopy (TEM), we adapted methods from Lange et al. (2009). We infected *E. postvittana* larvae and prepared tissues at different times to avoid any possible cross contamination. Although primary spores were observed in early stage infections, images for all stages were from late-stage infections and represented the second sporulation cycle (S2) (see Vavra et al., 2006b). For each isolate we dissected fifth instar larvae that had been infected in the second stadium and fixed infected mid-gut tissues for 1 h in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The fixed tissues were rinsed in 0.1 M sodium cacodylate buffer three times for 5 min and post-fixed in 1% aqueous osmium tetroxide and 1.6% potassium ferricyanide in 0.1 M sodium cacodylate buffer for 30 min. Post-fixed tissues were rinsed three times with 0.1 M sodium cacodylate buffer (5 min rinses) followed by three 10-min rinses with DI water. The tissues were then en bloc-stained with 0.5% aqueous uranyl acetate for 30 min in the dark and rinsed a further three times in DI water (5 min rinses). After staining, the tissues were dehydrated through an ascending acetone series (7 min in each of 35%, 50%, 70%, 80%, 95%), followed by 100% acetone for three times (7 min each). The tissues were then infiltrated through three gradients (15 min in each of 2:1, 1:1, 1:2) of acetone: Epon resin (23.5 g Eponate 12, 12.5 g dodecenyl succinic anhydride, 14 g nadic methyl anhydride, and 0.75 ml dimethylbenzylamine as the accelerator) followed by three times (15 min each) of pure resin. Tissues were agitated at all steps and embedded into molds with pure resin and accelerator at 60 °C for 72 h. Thin 70 nm sections were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with 2% aqueous uranyl acetate and lead citrate, and examined with a Tecnai 12 transmission electron microscope.

2.5. DNA extraction, sequencing and phylogenetic analysis

DNA was extracted from the *N. fumiferanae postvittana* isolates using the modified Chelex method (Walsh et al., 1991; Cordes et al., 2012). Purified spores (approximately 10^4–10^5) were added to 25 μl Chelex buffer composed of 10% Chelex 100 resin (BioRad), 5% tween20, and 1 μg/μl proteinase K, in a 200 μl PCR tube. The Chelex/spore mixtures were vortexed for 15 s at maximum speed and transferred into a thermocycler (BioRad iCycler). The mixtures were treated at 56 °C for 120 min, 95 °C for 30 min, and 4 °C for 1 min. The tubes were then centrifuged at 13,000 g for 10 min. Supernatants were stored at −20 °C until use.

We amplified fragments of three loci (ITS, SSU, and RPB1) that are commonly used for microsporidian species identification using standard PCR protocols. PCR reactions were conducted on a BioRad iCycler using the following conditions. After an initial denaturation step at 95 °C for 2 min, fragments were amplified using 40 cycles: 95 °C for 30 s, the fragment specific annealing temperature for 30 s (50 °C or 51 °C: Supplementary Table S1), and 72 °C for the fragment specific initial elongation time. A final elongation for 7 min at 72 °C was used. Primers and PCR conditions for amplification of each fragment are presented in Table S1. PCR reactions used 1 unit (0.2 μl) Taq DNA polymerase with 5 μl standard buffer, 1 μl dNTP mix, 1 μl DNA solution (supernatant from the Chelex method), and 1 μl of each 10 nM primer (100 nM in the case of the degenerate primers, Rpb1f1/Rpb1r1); in a 50 μl reaction. PCR products were run on a 1.5% agarose gel electrophoresis with 1 × TAE buffer and stained in ethidium bromide solution (1 ppm). PCR products with a single band and meeting estimated amplicon sizes in the gel electrophoresis were cleaned using ExoSAP-IT, and sequencing was performed with an ABI 3730 DNA analyzer with BigDye using concentrations and protocols according to the manufacturer's instructions. Sequences were submitted to GenBank under accession numbers KT020736 and KT020735 for ITS-SSUrRNA and RBP1 respectively.

The ITS region of the reversed rRNA gene arrangement (LSU–ITS–SSU) found in the *N. bombycis* group tends to be highly polymorphic (Huang et al., 2004), particularly in the center of the ITS region (~200 bp) (Ironside, 2013). As the ITS region adjacent to LSU was 100% identical to all published *Nosema* species having the reversed rRNA arrangement we focused on the more variable region adjacent to SSU. In addition, because the primers for ITS and SSU amplified fragments that had overlapping sequences,

these two regions were assembled into a single continuous sequence for the phylogenetic comparison (ITS–SSU).

The ITS–SSU and RPB1 fragments amplified from our *N. fumiferanae postvittana* isolate were compared to other available sequences from the genus *Nosema* and *Vairimorpha* that have been published in GenBank. Sequences were not consistently available for both markers and so the species representation differed for each marker (Table S3). An alignment for each locus was constructed using Clustal X version 2.0 (Larkin et al., 2007). The alignment was adjusted by eye and then truncated. Phylogenetic relationships between amplified and published sequences were reconstructed based on maximum likelihood using MEGA version 6.0 (Tamura et al., 2013) for each alignment under the GTR model with estimated gamma distribution and proportion of invariable sites following the methods of James et al. (2006). Support for each node was estimated using 1000 bootstrap replicates. To find a suitable outgroup for the ITS–SSU analysis, we noted that the non-coding region adjacent to the 5' end of *N. ceranae* SSU (Huang et al. (2008); GenBank accession # EF091879) shared 48–57% similarity with the non-polymorphic ITS region of *Nosema* species that have the reversed rRNA gene arrangement (Fig. S1). We hypothesized that the fragments are homologous and used *N. ceranae* as the outgroup species in the phylogenetic analysis of the ITS–SSU sequence as *N. ceranae* does not belong to the 'true *Nosema*' group (characterized by the reversed rRNA gene arrangement). *Encephalitozoon* species are not an appropriate outgroup for analysis of the ITS–SSU sequence since the identical non-coding region next to the 5' end of SSU had extremely low similarity. However, as the phylogenetic analysis of RPB1 concerns only one gene region, the arrangement of genes did not affect our selection of outgroups and the RPB1 gene of *N. ceranae* is too similar for this species to be used as an outgroup in this analysis. Thus, two *Encephalitozoon* species were used as the outgroup in the phylogenetic analysis of RPB1 because these are well studied species and are known to be distinct from *Nosema* species (Vossbrinck and Debrunner-Vossbrinck, 2005). The computed output was visualized using MEGA version 6.0 (Tamura et al., 2013).

2.6. Statistical analysis

Statistical analyses were carried out using R version 3.1.2 (R Core Team, 2014). A *t*-test and a Wilcoxon signed-rank test were used to compare the dimensions of fresh mature spores and the number of polar filament coils from the TEM images. All other analyses used generalized linear models (GLMs). Treatment (healthy or infected) and gender (male or female) were used as explanatory variables for analyzing each of the larval and pupal life history performance measurements, while treatment was the only explanatory variable for analyzing the measurements of adult female performance. As gender identity was assessed at pupation, gender could be used in analyzing larval development time, but could not be applied to larval survivorship. Analysis of daily fecundity, and egg hatch was confined to those females that successfully oviposited. Full models that included explanatory variables and two-way interactions were used initially, and stepwise model simplification was performed manually using likelihood-ratio tests (χ^2) in the absence of overdispersion and *F* tests to incorporate an empirical scale parameter in the presence of overdispersion (Crawley, 2013). Only significant interactions are presented in the results. Standard link functions were used for the GLMs and error distributions were selected to best represent the measurement variables analyzed (Gaussian for continuous variables, Poisson for counts, binomial for proportions). We ensured that error distributions were appropriate by inspecting plots of residuals or standardized deviance versus predicted values, and normal quantile plots (Crawley, 2013).

3. Results

3.1. Pathology and vertical transmission

All statistical analyses of life table measurements from *E. postvittana* larvae that were either uninfected (healthy) or infected with *N. fumiferanae postvittana* are presented in Table 1. Larval development time (days) was longer for infected females and males (21.50 ± 0.66 SE, 19.32 ± 0.58 SE respectively) compared to healthy females and males (20.33 ± 0.40 SE, 17.27 ± 0.30 SE respectively). Larval and pupal survivorships were lower for infected individuals compared to healthy individuals (Fig. 1A & B). Pupal fresh weight (mg) was greater for infected females and males (52.43 ± 1.52 SE, 30.74 ± 0.54 SE) compared to healthy females and males (49.56 ± 0.91 SE, 28.55 ± 0.53 SE). Pupal development time was slightly shorter for females (9.84 ± 0.15 SE) compared to males (10.83 ± 0.13 SE), but did not differ significantly between infected and healthy treatments. The percentage of females did not differ significantly between infected (43 ± 5 SE) and healthy (47 ± 5 SE) treatments. A significantly lower proportion of infected females eclosed (73 ± 8% SE) oviposited compared to the healthy females (93 ± 4% SE). Infected females also had reduced longevity, daily fecundity, and percent egg hatch compared to healthy females (Fig. 2A–C).

From the 17 infected *E. postvittana* females that survived to reproduce, 67 ± 6% (SE, $n = 68$) of the dissected egg masses collected on days 2, 6 and 10 showed visible signs of infection (spores were present) and thus confirmed vertical transmission. However, as we did not surface sterilize the eggs before dissection, it was not possible to determine whether the presence of spores was due to transovarial transmission at this stage. More than 10^4 spores per µl were counted in each of the ovipositing females at time of death ($2.2 \times 10^8 \pm 3.3 \times 10^7$ SE). There was no effect of female age (2, 6, or 10 days) (GLM (binomial), $\chi2 = 3.51$, $df = 2$, $P = 0.17$), female spore load (GLM (binomial), $\chi2 = 0.64$, $df = 1$, $P = 0.64$), or egg mass size (GLM (binomial), $\chi2 = 0.06$, $df = 1$, $P = 0.81$) on the prevalence of infection in egg masses. Of the small number of first instar larvae that hatched from eggs collected on days 2, 6 and 10, mature spores were present in 100 ± 0% (SE, $n = 12$), confirming transovarial transmission.

Table 1
Statistical analysis of the effect of infection by *Nosema fumiferanae postvittana* on the life history performance of *Epiphyas postvittana*.

Model	N^a (H, I)	GLM family, statistic	*df*	*P*
Larval survivorship	101, 95	binomial, $\chi2$		
Treatment		4.71	1	0.03
Larval development time	98, 85	Poisson, $\chi2$		
Treatment		6.36	1	0.01
Gender		16.38	1	<0.001
Pupal sex-ratio	98, 85	binomial, $\chi2$		
Treatment		0.09	1	0.76
Pupal fresh weight (mg)	98, 85	gaussian, *F*		
Treatment		8.13	1, 180	0.01
Gender		586.75	1, 180	<0.001
Pupal survivorship	98, 85	binomial, $\chi2$		
Treatment		13.45	1	<0.001
Gender		1.24	1	0.27
Pupal development time	97, 73	Poisson, $\chi2$		
Treatment		2.67	1	0.10
Gender		4.01	1	0.05
Female longevity	46, 33	Poisson, $\chi2$		
Treatment		29.26	1	<0.001
Oviposition	46, 33	binomial, $\chi2$		
Treatment		6.45	1	0.01
Daily fecundity	46, 33	Poisson, $\chi2$		
Treatment		41.88	1	<0.001
Egg hatch	43, 23	binomial, $\chi2$		
Treatment		1124.1	1	<0.001

[a] Number of healthy (H) and infected (I) individuals.

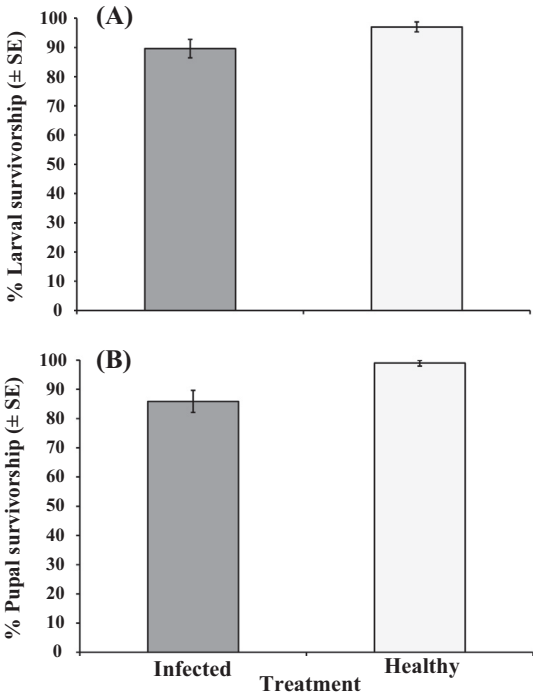

Fig. 1. Mean (±SE) larval survivorship (A) and pupal survivorship (B) for *Epiphyas postvittana* infected by *Nosema fumiferanae postvittana*, compared to healthy individuals.

3.2. Tissues infected and morphology

Observations of inoculated fifth instar *E. postvittana* larvae revealed that infection began in the midgut. Spores were observed in the midgut, Malphigian tubules and the silk glands (3 dpi), followed by the hemolymph (8 dpi), the fat body (8 dpi), and finally the developing gonads (10 dpi). All major organs were infected with spores at 10 dpi. Mature spores of both *N. fumiferanae* isolates were elongate ellipsoidal and highly refractive under phase contrast. Fresh mature spores of *N. fumiferanae postvittana* (Fig. 3A) measured 3.9 (±0.1 SE) × 1.8 (±0.1 SE) μm and those of *N. fumiferanae* measured 3.8 (±0.1 SE) × 1.9 (±0.0 SE) μm ($n = 10$ for each isolate) and did not differ significantly between isolates (*t*-test, $t_{length} = 0.55$, $P = 0.59$; $t_{width} = 0.79$, $P = 0.44$).

Images of the life cycle stages and ultrastructure of the two microsporidian isolates, *N. fumiferanae postvittana* and *N. fumiferanae* are presented in Figs. 4 and 5 respectively. All stages were diplokaryotic and from the second sporulation cycle. *N. fumiferanae postvittana* had significantly more polar filament coils (Wilcoxon signed-rank test, $V = 78.5$, $n = 41$, $P = 0.001$) arranged in a row (range 12–15, mean 14.32 ± 0.20 SE, $n = 20$, Fig. 4D, E) than *N. fumiferanae* (range 11–15, mean 12.86 ± 0.30 SE, $n = 21$, Fig. 5D and E). Polar filament coils were isofilar and arranged in single rows. The mature spores of both isolates possess a horseshoe shaped anchoring disk (relatively long arms), a lamellar polaroplast (Fig. 4D, E and Fig. 5D, E), and thick endospore. The meronts (Figs. 4A and 5A) were nested in the cytoplasm of the host cell, and were limited by a plasmalemma, which thickened at the sporont stage (Figs. 4B and 5B). The cytoplasm of the meronts, sporonts and sporoblasts contained ribosomes, cisternae of the rough and smooth endoplasmic reticulum and Golgi vesicles. Free ribosomes appeared in the meront and sporont stages, but were bound in the sporoblast and mature spores (Figs. 4 and 5).

3.3. Sequence comparison and phylogenetic analysis

We amplified a 110 base pair (bp) fragment of ITS, a 1178 bp fragment of SSU and a 562 bp fragment of RPB1 from purified *N. fumiferanae postvittana* spores isolated from three host individuals of *E. postvittana*. The fragment sequences of ITS and SSU we obtained were identical from the three host individuals and were

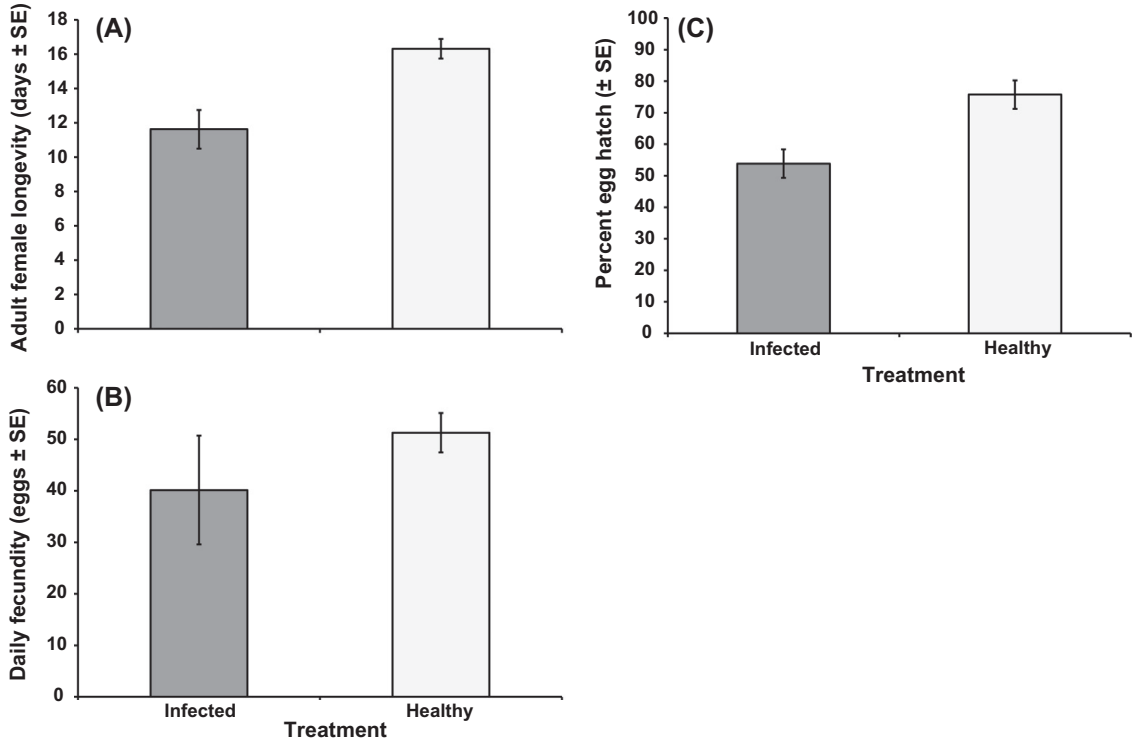

Fig. 2. Mean (±SE) adult longevity (A), daily fecundity (B), and the percent egg hatch (C) for *Epiphyas postvittana* infected by *Nosema fumiferanae postvittana*, compared to healthy individuals.

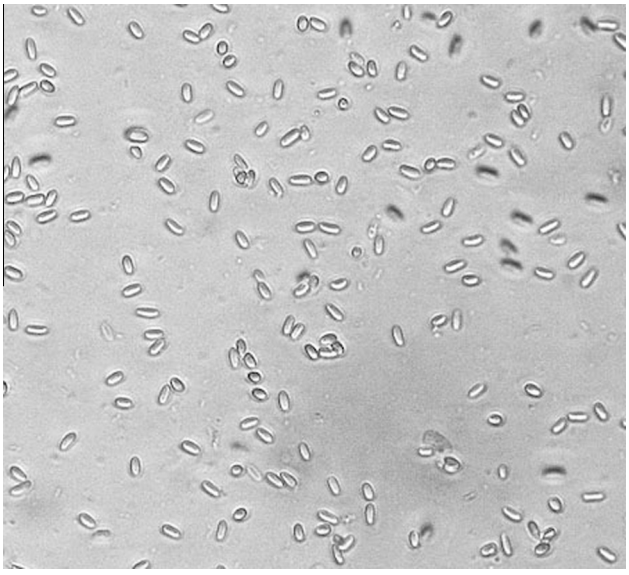

Fig. 3. Light micrograph of *N. fumiferanae postvittana* spores (400× phase contrast). Mean spore length × width: 3.9 (±0.1 SE) × 1.8 (±0.1 SE) μm.

highly similar to published sequences for *N. fumiferanae,* with only 6 bp differences observed between the isolates for both loci (two insertions and one polymorphic site for SSU, and three polymorphic sites for ITS). Sequencing of the forward primer of RPB1 failed, so our results are based on the sequences from the reverse primer only. No notable polymorphisms were found within sequences from RPB1, and *N. fumiferanae postvittana* shared 97% sequence similarity (12 bp differences) to published sequences from *N. fumiferanae.* These 12 bp differences corresponded to 10 synonymous and 2 non-synonymous mutations.

Phylogenetic reconstructions for ITS–SSU and RPB1 are presented in Figs. 6 and 7, respectively. Our reconstruction for ITS–SSU only included sequences from *Nosema* species, but we identified three well-supported clades (>91 bootstrap probability [BP]), with *N. ceranae* as the outgroup. One clade included published sequences for *N. philosamiae* and *N. antheraeae* (92 BP), a second clade included the sequence from *N. fumiferanae postvittana*, the published sequence for *N. fumiferanae,* and sequences from two unidentified *Nosema* species isolated from other *Choristoneura* hosts (93 BP), and the third clade included sequences from *N. bombycis, N. heliothidis, N. spodopterae*, and three sequences from unidentified *Nosema* spp. (91 BP). In general, the results from the reconstruction of RPB1 showed strong phylogenetic structure with several well-supported clades and outgroup (*Encephalitozoon* spp. [100 BP]). One clade (78 BP) included published sequences for *N. granulosis* and *V. cheracis,* another clade (100 BP) included sequences from *N. apis, N. ceranae*, *V. disparis*, and *V. necatrix*, and a third clade (99 BP) included the sequence from *N. fumiferanae postvittana, N. fumiferanae,* as well as published sequences from *N. antheraeae, N. bombycis*, *N. tyriae* and two unidentified *Nosema* species isolated from other *Choristoneura* hosts. Phylogenetic reconstructions for both loci confirm the relatedness of *N. fumiferanae postvittana* to *N. fumiferanae* (Table S2).

4. Discussion

Spores of *N. fumiferanae postvittana* were present in all tissues of fifth instar larval hosts 10 days post inoculation with 10^3 spores. Larval mortality was 7% higher for individuals infected as second instar larvae and mortality was 13% higher in infected pupae compared to healthy individuals. Furthermore, the pupation period was two days longer for infected individuals than for healthy individuals. Although males developed faster than females, we did not find a differential effect of gender on mortality. Adult females that were inoculated as second instar larvae died five days earlier, produced 47% fewer eggs in their lifetime, and experienced 22% lower egg hatch than healthy females. The majority of egg masses (68%) and all hatched larvae produced by infected females confirmed vertical transmission. These observations of systemic tissue infection, moderate pathology and transmission of *N. fumiferanae postvittana* from infected adults to their offspring, support the

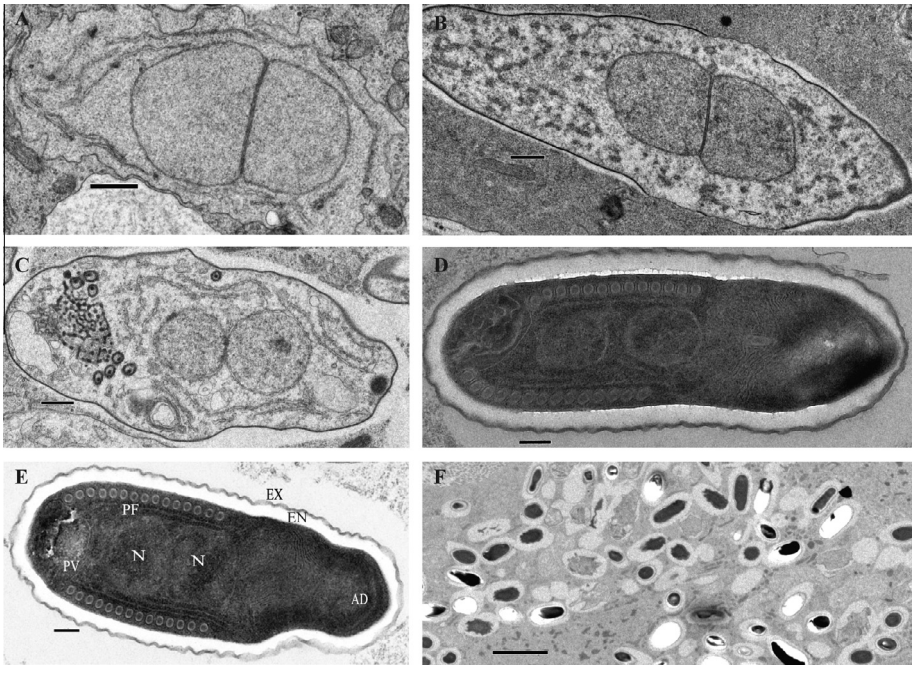

Fig. 4. Transmission electron micrographs of developmental stages of *Nosema fumiferanae postvittana* in the midgut tissues of the host, *Epiphyas postvittana.* (A) Binucleate meront, bar = 0.5 μm. (B) Sporont, bar = 0.5 μm. (C) Sporoblast; bar = 0.5 μm. (D) Mature spore, bar = 0.2 μm. (E) Mature spore, bar = 0.2 μm; AD = anchoring disk; EX = exospore; EN = endospore; N = nucleus; PF = polar filament; PV = polar vacuole. (F) All developmental stages; bar = 5 μm.

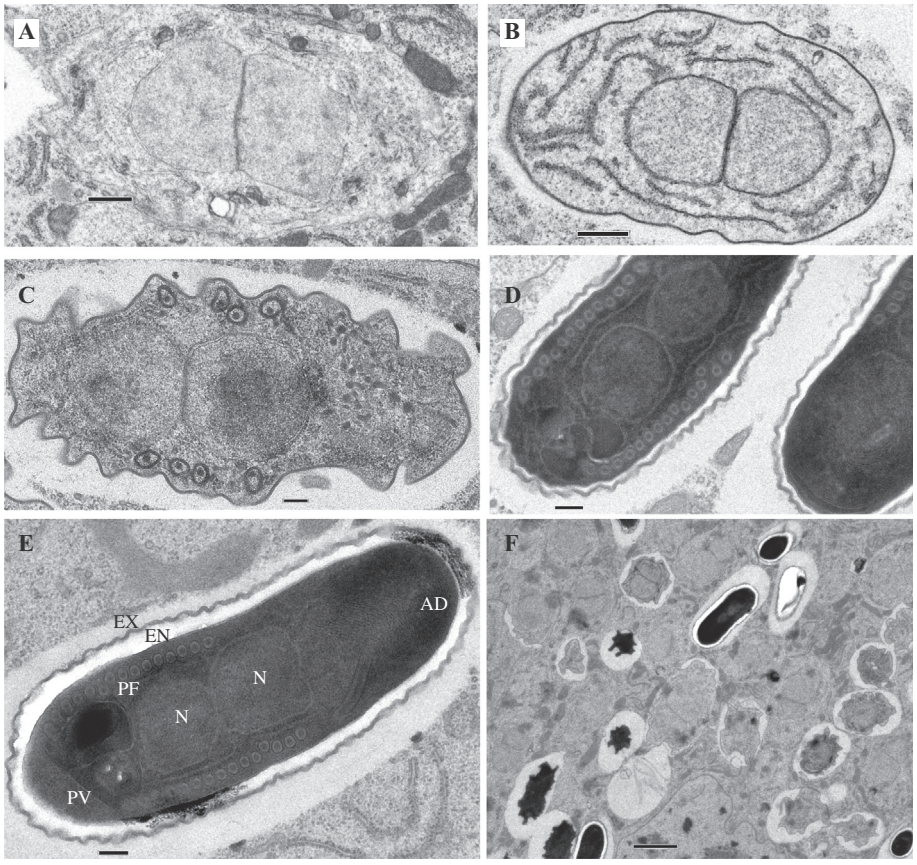

Fig. 5. Transmission electron micrographs of the developmental stages of *Nosema fumiferanae* in the midgut tissues of the host, *Epiphyas postvittana*. (A) Binucleate meront; bar = 0.5 μm. (B) Sporont; bar = 0.5 μm. (C) Sporoblast; bar = 0.2 μm. (D) Mature spore, bar = 0.2 μm. (E) Mature spore, bar = 0.2 μm; AD = anchoring disk; EX = exospore; EN = endospore; N = nucleus; PF = polar filament; PV = polar vacuole. (F) All developmental stages; bar = 2 μm.

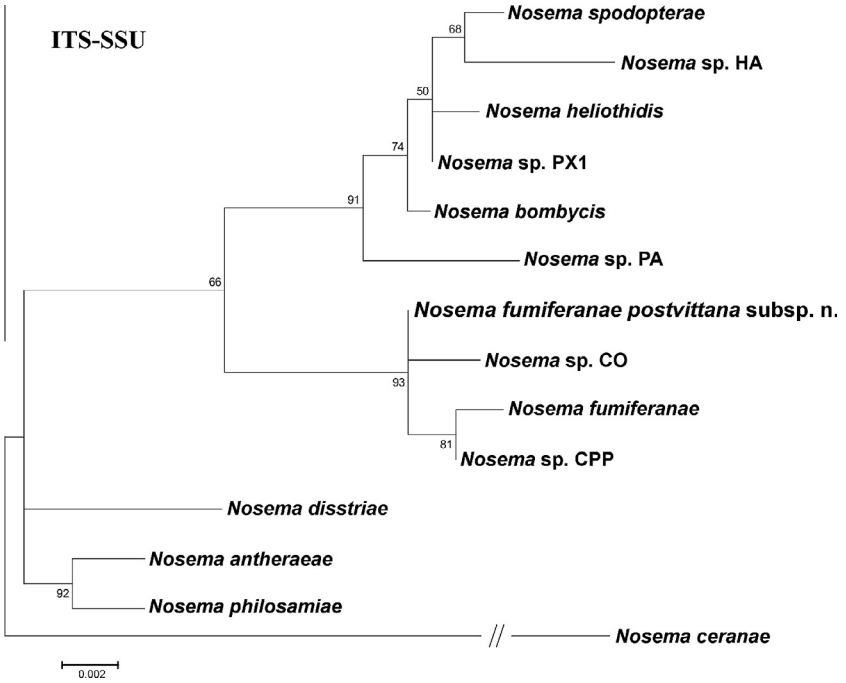

Fig. 6. Phylogenetic tree of non-polymorphic ITS–SSU gene nucleotides. The numbers are the supporting values of the nodes from 1000 bootstrap replicates. *Nosema ceranae* was used as the outgroup for rooting the ITS–SSU tree (with a shortened branch length to fit the figure). GenBank accession numbers are in Table S3.

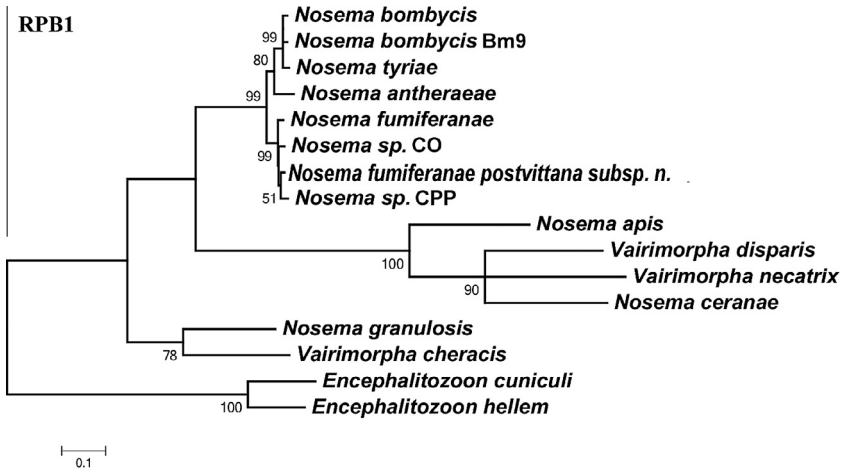

Fig. 7. Phylogenetic tree of RPB1 gene nucleotides. The numbers are the supporting values of the nodes from 1000 bootstrap replicates. *Encephalitozoon* species were used as the outgroup for rooting the RPB1 tree. GenBank accession numbers are in Table S3.

strategy of a low virulence pathogen that develops slowly, allows its host to continue development to the adult stage and is maintained in the host population by vertical transmission (Anderson and May, 1981; Solter, 2006).

The pathology and transmission of *N. fumiferanae postvittana* with respect to *E. postvittana* is similar to that reported for *N. fumiferanae* for *C. fumiferana* (Thomson, 1957; Bauer and Nordin, 1989). Vertical transmission in *C. fumiferana* was also found to result in 100% infection of hatching larvae in a study by Bauer and Nordin (1989). However, van Frankenhuyzen et al. (2007) found vertical transmission to be dependent on infection intensity in the parent female, with 100% transmission to eggs and larvae occurring only when females contained more than $4.5–5 \times 10^5$ spores. Although we found variation in the extent of vertical transmission among parent females, we did not find a relationship with infection intensity. However, in our study, all ovipositing females contained over 10^4 spores per µl at the time of death.

One difference in the pathology of the *Nosema* isolates from *E. postvittana* and *C. fumiferana* appears to be the effect of infection on host pupal fresh weight. Thomson (1957) and Bauer and Nordin (1989) found that infected *C. fumiferana* pupae were significantly smaller than healthy pupae. Reduced pupal fresh weight was consistent for larvae infected via vertical transmission and for larvae infected as fourth and fifth instars (Bauer and Nordin, 1989). These observations contrast with our results from *E. postvittana* larvae infected as second instars, for which pupal fresh weight was greater than that of healthy individuals for both males and females. We did not measure the dimensions of the *E. postvittana* pupae, so it remains unknown whether the increased fresh weight of infected pupae was due to additional growth of the host larvae (which required a slightly longer time period to complete their development), or to induced cell hypertrophy (Vavra and Lukes, 2013).

In addition to the similarities in host pathology between *N. fumiferanae* and *N. fumiferanae postvittana*, both isolates also exhibit the highly conserved reversed arrangement of rRNA gene subunits, in which the sequence order is LSU–ITS–SSU (Huang et al., 2004). This further supports the suggestion that the genus *Nosema* should be reserved for species that have the reversed arrangement of rRNA gene subunits (Huang et al., 2004; Tsai et al., 2005; Kyei-Poku et al., 2008, 2012). The SSU fragment is very similar among *Nosema* species, with 98–100% similarity among species in the *N. bombycis* group (Tsai et al., 2005; Kyei-Poku et al., 2008). We found only three nucleotide differences in the sequences from *N. fumiferanae postvittana*, *N. fumiferanae*, *N. bombycis*, and *Nosema* sp. CPP (isolated from *Choristoneura pinus pinus* Freeman), and eight nucleotide differences from *Nosema* sp. CO (isolated from *Choristoneura occidentalis* Freeman). Similar differences in SSU sequences have been found for other *Nosema* species that have non-overlapping host ranges (Tsai et al., 2005).

In both of our phylogenetic reconstructions (ITS–SSU and RPB1), we found that our isolate, *N. fumiferanae postvittana,* was placed within the *N. bombycis* group and within the *N. fumiferanae* species complex along with two other closely related isolates (*Nosema* sp. CPP and *Nosema* sp. CO) from *Choristoneura* hosts (Kyei-Poku et al., 2008). In general, we also found that the topologies of both reconstructions were broadly congruent in that the *N. bombycis* group formed a distinct clade in both analyses, though this clade was not well supported in either reconstruction. One difference between the reconstructed topologies was the placement of *N. antheraeae* which may have been a result of the relatively short branch lengths observed between individuals in the ITS–SSU analysis (as suggested by the lack of support), the independent evolutionary histories of these two loci, and/or because these sequences were obtained from GenBank and may be products of species-level misidentification (Vilgalys, 2003). Despite these differences, our results demonstrate overall congruence between the reconstructions from these two markers and add further support to the growing use of RPB1 for both species-level and higher taxonomic analyses (Hirt et al., 1999; Cheney et al., 2001; Vavra et al., 2006a; Gisder and Genersch, 2013; Luo et al., 2014).

According to our morphological and phylogenetic analyses, we consider the isolate, *N. fumiferanae postvittana*, to be part of the *Nosema fumiferanae* species complex, along with *Nosema* sp. CO and *Nosema* sp. CPP. Thus, *N. fumiferanae postvittana* is potentially a Nearctic species originating from resident tortricid hosts, and shares a very close evolutionary relationship with other members of the *N. fumiferanae* species complex isolated from North America forest habitats. Nevertheless, we cannot exclude the possibility that *N. fumiferanae postvittana* was carried by *E. postvittana* (also in the family Tortricidae) when it invaded California, as no studies of microsporidian infection of this host in Australia have been published.

The similarity of the ultrastructure images of both *N. fumiferanae* isolates further supports their relatedness, and their separation from *N. bombycis*. The ultrastructure images of *N. bombycis* (Sato et al., 1982) demonstrate a bulbous-shaped anchoring disk compared to the horseshoe shaped anchoring disk in the two *N. fumiferanae* isolates. The two rows of polar filament coils are also more symmetrical in the two *N. fumiferanae* isolates than in

N. bombycis. However, the mean number of polar filament coils in mature spores of *N. bombycis* was observed to be 12.21 (Sato et al., 1982), very similar to the 12.86 coils that we observed in spores of *N. fumiferanae*, but fewer than the 14.32 coils that we observed in spores of *N. fumiferanae postvittana* and the 14.71 coils observed for *Nosema* sp. M12 (Sato et al., 1982). It should be noted, however, that the polar filament coil counts were made from spores of *N. fumiferanae* infecting *E. postvittana* rather than *C. fumiferana*, the natural host of the isolate.

While we did not specifically test the host range of *N. fumiferanae postvittana*., we discovered that an egg parasitoid, *Trichogramma fasciatum* (Perk.) and a larval endoparasitoid, *Meteorus ictericus* Nees, were also susceptible to infection when reared in infected *E. postvittana* (Hopper, unpublished observations). This was not surprising as infections by microsporidia in parasitoids of infected hosts are not uncommon (Bjornson and Oi, 2014) and spores of *N. fumiferanae* were recovered from two larval parasitoids of *C. fumiferana*: *Apanteles fumiferanae* and *Glypta fumiferanae* (Thomson, 1958).

If the *N. fumiferanae* species complex is North American in origin, the acquisition of an indigenous microsporidium by *E. postvittana* may have occurred via horizontal transmission from a resident insect host (possibly a tortricid) or the intervention of a vector, such as parasitoids or birds. The ability of parasitoids to vector microsporidia to uninfected hosts is variable (Own and Brooks, 1986; Siegel et al., 1986; Hoch et al., 2000; Futerman et al., 2006; Simões et al., 2012; Saito and Bjornson, 2013), but birds can act as potential vectors by dispersing pathogens after consuming infected prey (Entwistle et al., 1978; Slodkowicz-Kowalska et al., 2006).

In conclusion, from the pathology, morphology and sequence results, we cannot determine with certainty whether *N. fumiferanae postvittana* is a novel species. We suggest that it belongs to the *N. fumiferana* species complex that also includes *Nosema* sp. CO and *Nosema* sp. CPP. Although we cannot determine the origin of this isolate without further exploration of the host in its native range, this novel pathogen potentially contributes further to the biotic resistance that *E. postvittana* has experienced from other resident natural enemies in California (Wang et al., 2012; Bürgi and Mills, 2014; Bürgi et al., 2015).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2016.01.001.

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