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ORIGINAL ARTICLE

Nosema maddoxi sp. nov. (Microsporidia, Nosematidae), a Widespread Pathogen of the Green Stink Bug *Chinavia hilaris* (Say) and the Brown Marmorated Stink Bug *Halyomorpha halys* (Stål)

Ann E. Hajek[a], Leellen F. Solter[b], Joseph V. Maddox[b], Wei-Fone Huang[c], Alden S. Estep[d,e], Grzegorz Krawczyk[f], Donald C. Weber[g], Kim A. Hoelmer[h], Neil D. Sanscrainte[e] & James J. Becnel[e]

a Department of Entomology, Cornell University, Ithaca, New York, 14853-2601, USA
b Illinois Natural History Survey, Prairie Research Institute, University of Illinois, Champaign, Illinois 61820, USA
c College of Bee Science, Fujian Agriculture and Forestry University, Fujian, Fuzhou 350002, China
d Navy Entomology Center of Excellence, Naval Air Station, Jacksonville, Florida 32212, USA
e Center for Medical, Agricultural and Veterinary Entomology – CMAVE (USDA, ARS), Gainesville, Florida 32608, USA
f Department of Entomology, Fruit Research and Extension Center, Pennsylvania State University, Biglerville, Pennsylvania 17307, USA
g USDA ARS Invasive Insect Biocontrol and Behavior Laboratory, Beltsville, Maryland 20705, USA
h USDA ARS Beneficial Insect Introduction Research Unit, Newark, Delaware 19713, USA

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Correspondence
A.E. Hajek, Department of Entomology, Cornell University, 129 Garden Avenue, Ithaca, NY 14853-2601, USA
Telephone number: +1-607-254-4902;
FAX number: +1-607-255-0939;
e-mail: aeh4@cornell.edu

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ABSTRACT

We describe a unique microsporidian species that infects the green stink bug, *Chinavia hilaris;* the brown marmorated stink bug, *Halyomorpha halys;* the brown stink bug, *Euschistus servus;* and the dusky stink bug, *Euschistus tristigmus*. All life stages are unikaryotic, but analysis of the consensus small subunit region of the ribosomal gene places this microsporidium in the genus *Nosema*, which historically has been characterized by diplokaryotic life stages. It is also characterized by having the reversed arrangement of the ribosomal gene (LSU –ITS- SSU) found in species within the "true *Nosema*" clade. This microsporidium is apparently Holarctic in distribution. It is present in *H. halys* both where it is native in Asia and where it is invasive in North America, as well as in samples of North American native *C. hilaris* collected prior to the introduction of *H. halys* from Asia. Prevalence in *H. halys* from mid-Atlantic, North America in 2015–2016 ranged from 0.0% to 28.3%, while prevalence in *C. hilaris* collected in Illinois in 1970–1972 ranged from 14.3% to 58.8%. Oral infectivity and pathogenicity were confirmed in *H. halys* and *C. hilaris*. Morphological, ultrastructural, and ecological features of the microsporidium, together with a molecular phylogeny, establish a new species named *Nosema maddoxi* sp. nov.

MICROSPORIDIA are common pathogens of insects, but few species have been reported from hemipteran hosts compared to the other major insect orders, particularly the Lepidoptera, Diptera, and Coleoptera (Becnel and Andreadis 2014). Approximately 20 species of microsporidia have been reported from hemipteran hosts (Table 1) with only two species from hosts in the stink bug family, Pentatomidae, both undescribed. The undescribed pentatomid microsporidium isolated from the green stink bug, *Chinavia* (*Acrosternum*) *hilaris* (Say), in the North American Midwest was reported in a brief abstract (Maddox 1979), but no formal description or assignment to a genus has been published.

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is a polyphagous pest of beans, flowers, and fruit crops and other trees in Japan, China, and Korea and a nuisance pest in houses (Lee et al. 2013). It was first recorded in North America in Allentown, Pennsylvania in

Table 1. Microsporidia reported from aquatic and terrestrial Hemiptera

Hemipteran host [family]	Microsporidium	References	GenBank acc.
Aquatic hosts			
Aquarius najas (de Geer) [Gerridae]	*Toxoglugea gerridis* Poisson 1941	Poisson (1941)	
Sigara lateralis (Leach) [Corixidae]	*Becnelia sigarae* Tonka and Weiser 2000	Tonka and Weiser (2000)	
Nepa cinerea L. [Nepidae]	*Chapmanium nepae* (Lipa 1966)	Lipa et al. (1975)	
	Nosema bialoviesianae Lipa 1966 and *Thelohania nepae* Lipa 1966	Lipa (1966a)	
	Nosema nepae Poisson 1928	Poisson (1929)	
Notonecta viridis Delc. [Notonectidae]	*Toxoglugea mercieri* (Poisson 1924)	Poisson (1924)	
Velia currens (Fab.) [Veliidae]	*Nosema veliae* Poisson 1929	Poisson (1929)	
Omania coleoptrata Horv. [Omaniidae]	*Nosema omaniae* Diarra & Toguebaye 1995	Diarra and Toguebaye (1995)	
Terrestrial hosts			
Aphis rumicis L. [Aphididae]	*Toxoglugea fanthami* Weiser 1961	Fantham and Porter (1958) and Weiser (1961)	
Blissus leucopterus hirtus Mont. [Lygaeidae]	*Nosema blissi* Liu & McEwen 1977	Liu and McEwen (1977)	
Halyomorpha halys (Stål) [Pentatomidae]	*Nosema* sp.[b]	Sparks et al. (2014)	PRJNA242849
Chinavia hilaris (Say)[c] [Pentatomidae]	*Microsporidium* sp.[d]	Maddox (1979)	
Cimex hemipterus Fab. [Cimicidae]	*Microsporidium* sp. (previously *Nosema adiei* Christophers 1922)	Christophers (1922), Shortt and Swaminath (1924), and Sprague (1977)	
Dysdercus peruvianus (Guerin-Meneville) [Pyrrhocoridae]	*Microsporidium* sp.[d]	Milano et al. (1999)	
Empoasca fabae (Harris) [Cicadellidae]	*Nosema empoascae* Ni, Backus & Maddox 1995	Ni et al. (1995)	DQ996238.1
Graphosoma italicum Müll. [Pentatomidae]	*Microsporidium* sp.[e]	Galli-Valerio (1924) and Weiser (1961)	
Lygus lineolaris (Palisot de Beauvois) [Miridae]	*Nosema* sp.[b]	Musser et al. (2012)	JQ713130.1
Boisea trivittata (Say) (=*Leptocoris trivittatus* (Say)) [Rhopalidae]	*Nosema lepticordis* Lipa 1966	Lipa (1966b)	
Megacopta cribraria (Fab.) [Plataspidae]	*Nosema* sp. MC	Xing et al. (2014)	KJ494249
Panstrongylus megistus (Burmeister) [Reduviidae]	*Octosporea carloschagasi* Kramer 1972	Kramer (1972)	
Pyrrhocoris apterus (L.) [Pyrrhocoridae]	*Nosema pyrrhocoridis* Lipa 1977	Lipa (1977)	

[a]Described by Lipa (1966a) as a mixed infection of *Nosema bialoviesianae* and *Thelohania nepae.* Possible *Vairimorpha* sp.
[b]Assigned as a *Nosema* sp. based on molecular characterization only with no additional information.
[c]Synonyms: *Pentatoma hilaris, Nezara hilaris, Acrosternum hilare, Acrosternum hilaris, Chinavia hilare* [Family: Pentatomidae].
[d]Noted as *Microsporidium* species but not further characterized.
[e]Described as *Nosema graphosomae* Galli-Valerio 1924 (Galli-Valerio 1924) but Weiser (1961) stated that the description of this gut microsporidium is not sufficient for an identification of this species.

1996 (Hoebeke and Carter 2003) and has since spread to at least 43 states in the U.S., and four Canadian provinces (Northeastern IPM Center 2016) and to western Europe (Haye et al. 2015). *Halyomorpha halys* feeds on more than 100 plants in 45 families, and invasive populations have caused significant damage in apples and other tree fruit, row, and vegetable crops and ornamental plants. With the relatively recent invasion of *H. halys* into the U.S., there has been interest in identifying and testing natural control agents for stink bugs, requiring mass rearing of laboratory colonies. Investigations to determine the cause of decline of a *H. halys* colony used to rear parasitoids led to the isolation of a microsporidium as the causative agent.

We compared the microsporidium from the *H. halys* colony to isolates of the undescribed microsporidium from the native *C. hilaris* collected in Illinois between 1968 and 1972 and to isolates from *H. halys* and native stink bugs collected from the mid-Atlantic U.S. in 2015–2016. We report here that all of these isolates are the same species. In addition, this microsporidium was also recovered from *H. halys* collected in Asia, confirming that it has a Holarctic distribution. Here, we provide detailed information on morphological, ultrastructural, and ecological features, and a molecular phylogeny based on analysis of the consensus small subunit (SSU) region of the ribosomal gene to describe this new species and establish the new taxon *Nosema maddoxi* sp. nov. The description contributes to a

better overall understanding of how the morphological and genetic features of this microsporidian clade have evolved.

MATERIALS AND METHODS

Sample collections

Collections of pentatomids examined for this study focused on the exotic brown marmorated stink bug, *H. halys*, and the North American native green stink bug, *C. hilaris,* although field-collected specimens of other native North American species were also examined. There were five sources of stink bugs examined by either light microscopy, electron microscopy, and sequencing or a combination of methods. Fresh tissue smears of live adults were examined for the presence of spores with phase contrast microscopy to identify microsporidian-infected individuals. In addition, approximately 20–25 adults of the egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), reared on infected *H. halys* (Source 2, below), were examined individually with fresh smears for detecting the presence of spores.

- Source 1 hosts were *C. hilaris* adults, field-collected from several counties in Illinois in 1970–1972. Prevalence data were based on light-trap collections in Monticello (Piatt County), Illinois, from May through October and have not been published previously. Specimens of *C. hilaris,* collected in Illinois in 1968 and stored in liquid nitrogen, were used for molecular studies.
- Source 2 was a declining colony (in 2012) of *H. halys* used to rear the egg parasitoid *T. japonicus* at the Department of Plant Industries (DPI, Gainesville, Florida). This *H. halys* colony originated from a colony maintained at the USDA ARS Beneficial Insects Introduction Research Unit, Newark, Delaware, that was initiated with locally collected material in northern Delaware.
- Source 3 included field samples of *H. halys* and mixed native stink bug populations, primarily *C. hilaris* and *Euschistus servus* (Say), sampled in August–October 2015 and 2016 at numerous sites in Pennsylvania, New York, Virginia, Maryland, and North Carolina. Only samples from Adams and Lancaster Cos., Pennsylvania, were used for developmental and molecular studies. These samples were collected by hand from foliage and pheromone traps, from a variety of habitats including orchards and soya bean fields.
- Source 4 was a declining colony of *H. halys* maintained at the University of Maryland and was used in transmission studies.
- Source 5 was alcohol-preserved specimens of *H. halys* collected from China and South Korea from April to July 2016 for use in molecular studies. From China, two adults were collected from Shandong Prov., Lantai Memorial Cemetery, eight adults from Shandong Prov., Fushan County, and seven adults and nine nymphs from Yunnan Prov., Yiliang City. From South Korea, five adults were collected from Gang-Won Prov., Seo-myeon Hongcheon-gun. All individuals were examined for patent microsporidian infections.

Pathogen development and ultrastructural studies

Air-dried tissue smears prepared from eggs, nymphs, and adults (Sources 1, 2, and 3) were fixed with methanol and stained with Giemsa (Becnel 2012). Spore measurements were obtained from both Giemsa-stained and fresh-mounted preparations using an image-splitting eyepiece (*C. hilaris* host; Source 1) and SPOT Imaging Software v4.7 (SPOT Imaging, Sterling Heights, MI) (*H. halys* host; Source 2).

For transmission electron microscopy (TEM), nymphs and adults (Sources 2 and 3) were dissected in 2.5% glutaraldehyde and held for a minimum of 2 h, postfixed in 2% osmium tetroxide for 1.5 h, dehydrated in an ethanol series to absolute acetone and embedded in epon-araldite (Becnel 2012), and thin-sectioned. Sections were post-stained with 2.5% uranyl acetate followed by lead citrate and viewed and photographed on a Hitachi H-600 (Hitachi America, Tarrytown, NY) electron microscope at 75 kV. Unpublished micrographs of vegetative stages and spores from Source 1 material (J.V.M., unpublished) were compared to similar stages from Source 2 and 3 material observed in this study, and no significant morphological differences were found. Therefore, micrographs presented in the figures are from Source 2 and 3 materials.

Sequencing and phylogeny

Genomic DNA (gDNA) was extracted from portions of individual stink bug homogenates (fresh, frozen, or alcohol-preserved specimens; Sources 2, 3, and 5) using the OmniPrep Kit (G-Biosciences, St. Louis, MO) as per the manufacturer's instructions, with the addition of boiling each sample for 15 min after homogenization followed by an RNAse incubation step. Samples were homogenized by bead beating at max speed for 2–3 min on a Mini-BeadBeater-24 (BioSpec, Bartlesville, OK) using 2.7 mm glass beads. Analysis of DNA purity was performed on a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA).

gDNA samples were probed with universal cytochrome c oxidase I (COI) primers (*LCO1490*, 5′-GGTCAACAAATCATAAAGATATTGG-3′ and *HCO2198*, 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) to provide host identification. Several universal primers targeting the microsporidian ribosomal SSU 16s were used to screen for microsporidia infection (*18f*, 5′- CACCAGGTTGATTCTGCCTGAC-3′; *1492r*, 5′-GGTTACCTTGTTACGACTT-3′; and *1537r*, 5′-TTATGATCCTGCTAATGGTTC-3′) (Ghosh and Weiss 2009). Amplicons from each primer set were purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA) and submitted for sequencing to Macrogen USA (Rockville, MD).

Additionally, full-length LSU-ITS-SSU was amplified using the primers *LS228f* (5′-GGAGGAAAAGAAACTAAC-3′) (Huang et al. 2004) and *1537r* from microsporidian-infected *H. halys*, *E. servus* and *C. hilaris* (~4 kb).

Gel-purified amplicons were inserted into pGEM T-easy vector (Promega, Madison, WI) and cloned using chemocompetent One Shot TOP10 cells (Invitrogen/Thermo Fisher Scientific, Waltham, MA) as per manufacturer's protocols. Full-length sequences from 7 to 9 clones from each individual were obtained using primers from Huang et al. (2004). Sequences were trimmed based on chromatogram analyses and assembled manually or by using cap3 (http://doua.prabi.fr/software/cap3), and BLASTn analysis was performed.

Small subunit sequences of the type species *Nosema bombycis* (Naegeli) and *Vairimorpha necatrix* (Pilley) were used for an initial BLAST search to identify close relatives. Outgroup sequences were chosen as the next nearest neighbour clade from a previously published phylogeny (Vossbrinck et al. 2014, see Table 6.2). The recently published SSU for *Rugispora istanbulensis* Bekircan et al. (KR704648.1) was included because of its phylogenetic position in the *Nosema* clade. All sequences (Table 2) were aligned in SeaView version 4 (Gouy et al. 2010) using the included MUSCLE algorithm. Truncated sequences were removed, unaligned excess sequence was trimmed from the ends and then the trimmed sequences were realigned. The resulting alignment (Fig. S1) was transferred into MEGA7.0 (Kumar et al. 2016), realigned and used for phylogenetic reconstruction. The HKY + G nucleotide substitution model was identified as the overall best fit in MEGA Maximum likelihood reconstruction, and 500 rounds of bootstrapping were performed using PhyML as instituted in the MEGA software.

Table 2. Accessions and hosts of microsporidian species used for phylogenetic analysis

Accession	Species name	Order	Family	Host species
Vairimorphidae				
DQ996241.1	***Vairimorpha necatrix***	**Lepidoptera**	**Noctuidae**	***Mythimna unipuncta***
AF426104.1	*Nosema carpocapsae*	Lepidoptera	Tortricidae	*Cydia pomonella*
U27359.1	*N. oulemae*	Coleoptera	Chrysomelidae	*Oulema melanopus*
EU219086.1	*N. thomsoni*	Lepidoptera	Tortricidae	*Choristoneura conflictana*
AF033316.1	*N. portugal*	Lepidoptera	Erebidae	*Lymantria dispar*
AF033315.1	*V. lymantriae*	Lepidoptera	Erebidae	*Lymantria dispar*
U11047.1	*N. vespula*	NA	NA	NA
DQ272237.1	*Thelohania disparis*	Lepidoptera	Erebidae	*Lymantria dispar*
JX268035.1	*N. pieriae*	Lepidoptera	Pieridae	*Pieris brassicae*
EU487251.1	*V.* sp. *CHW-2008a*	Lepidoptera	Bombycidae	*Ernolatia lida*
FJ481912.1	*N. ceranae*	Hymenoptera	Apidae	*Apis mellifera*
AF495379.1	*Oligosporidium occidentalis*	Mesostigmata	Phytoseiidae	*Metaseiulus occidentalis*
KC412706.1	*N. adaliae*	Coleoptera	Coccinellidae	*Adalia bipunctata*
U26534.1	*N. apis*	Hymenoptera	Apidae	*Apis mellifera*
KR704648.1	*Rugispora istanbulensis*	Coleoptera	Chrysomelidae	*Xanthogaleruca luteola*
Nosematidae				
L39111.1	***N. bombycis***	**Lepidoptera**	**Bombycidae**	***Bombyx mori***
FJ772435.1	*N. heliothidis*	Lepidoptera	Noctuidae	*Helicoverpa armigera*
AY747307.1	*N. spodopterae*	Lepidoptera	Noctuidae	*Spodoptera litura*
AJ012606.1	*N. tyriae*	Lepidoptera	Erebidae	*Tyria jacobaeae*
EU864526.1	*N. antheraeae*	Lepidoptera	Saturniidae	*Antheraea pernyi*
HM566196.1	*N. pyrausta*	Lepidoptera	Crambidae	*Ostrinia nubilalis*
AJ131646.1	*V. imperfecta*	Lepidoptera	Plutellidae	*Plutella xylostella*
KT020736.1	*N. fumiferanae*	Lepidoptera	Tortricidae	*Epiphyas postvittana*
EU267796.1	*V. ceraces*	Lepidoptera	Tortricidae	*Cerace stipatana*
AF240355.1	*Endoreticulatus* sp.	Lepidoptera	Bombycidae	*Bombyx mori*
KY783594–KY783624	*N. maddoxi* sp. nov.	Hemiptera	Pentatomidae	*Chinavia hilaris*
EU012490.1	*Micro.* sp. *ZYS-3*	Lepidoptera	Pieridae	*Pieris rapae*
AY960987.1	*N. plutellae*	Lepidoptera	Plutellidae	*Plutella xylostella*
HQ457431.1	*N. disstriae*	Lepidoptera	Lasiocampidae	*Malacosoma disstria*
HQ180013.1	*N. mylitta*	Lepidoptera	Saturniidae	*Antheraea mylitta*
AJ011833.1	*N. granulosis*	Amphipoda	Gammaridae	*Gammarus duebeni*
U26532.1	*N. furnacalis*	Lepidoptera	Crambidae	*Ostrinia furnacalis*
DQ996238.1	*N. empoascae*	Hemiptera	Cicadellidae	*Empoasca fabae*
KC412707.1	*N. chrysoperlae*	Neuroptera	Chrysopidae	*Chrysoperla carnea*
AF327408.1	*V. cheracis*	Decapoda	Parastacidae	*Cherax destructor*
KJ494249.1	*Nosema* sp. *MC*	Hemiptera	Plataspidae	*Megacopta cribraria*

Generic type species and hosts are in bold type. NA, Not available.

Infectivity and host specificity testing

Healthy *H. halys* were reared following methods described in Herlihy et al. (2014), and both rearing and bioassays were conducted at 25 °C and 50% RH. Two cadavers of adult *H. halys* killed by microsporidian infections (Source 4; verified with molecular analysis to be the same found in other sample groups) were homogenized individually in 1.5-ml tubes containing 500 μl distilled water using a microcentrifuge pestle. Spore density was quantified using a haemocytometer. The surfaces of two egg masses that were estimated to be 24 h from hatch were covered with 20 μl of one of two different spore suspensions: Bioassay 1: 3.84×10^4 spores/μl and Bioassay 2: 4.76×10^3 spores/μl. An additional egg mass was maintained as a control (26 eggs). After hatch, nymphs were monitored every 1–3 d, and any cadavers were held at −20 °C until diagnosis. All cadavers were individually macerated with a pestle in a 1.5-μl tube contained 50–500 μl distilled water (amount varying by instar), and a smear was observed at 400X under phase contrast microscopy to detect microsporidian spores as evidence of infection.

For host specificity testing in hemipterans, spores were purified by centrifugation of macerated abdominal tissues and suspended in water to a density of approximately 10^7 spores/ml. Droplets of suspension (100 μl) were fed to 20 or more individuals of potential host species, the pentatomids *C. hilaris, Podisus maculiventrus* (Say), *Euschistus variolarius* (Palisot de Beauvois), and *Nezara viridula* (L.), the lygaeid *Oncopeltis fasciatus* (Dallas), and the mirid *Lygus lineolaris* (Palisot de Beauvois). All hemipteran individuals were starved, and liquids were withheld for 24 h before being offered droplets. Individuals that did not feed on most or all of the droplet were discarded. The lepidopteran larvae, *Diacrisia virginica* (Fab.) (Erebidae), *Mythimna unipuncta* (Haworth) (Noctuidae), and *Helicoverpa zea* (Boddie) (Noctuidae), were fed 100 μl of a spore suspension containing approximately 10^6 spores spread on the surface of artificial diet in 30-ml plastic cups. Treated individuals were incubated for 2 wk at 22.5 °C, and then tissues were examined for infection under 400X phase contrast microscopy. Only species with both vegetative forms and spores of the microsporidium in the cells of tissues were considered to be infected.

RESULTS

Developmental studies

Infection with *Nosema maddoxi* sp. nov. in *C. hilaris* adults (Source 1) began in the mid-gut but infected epithelial cells were shed and newly formed cells were not infected in late-stage infections. Similar to *Vairimorpha* species and *Nosema lymantriae* (Vavra et al. 2006), the mid-gut epithelium did not appear to be the primary focus of infection. Tissues with high-spore loads included fat body, salivary glands, scent glands, and female reproductive tissues (oviduct, accessory glands, and the mucous surrounding the eggs). Malpighian tubules, testes, and mid-gut epithelia were less heavily infected, and embryos were not infected. Infection progressed from mid-gut epithelia to salivary and scent glands (~100- to 150-h postinoculation), then to fat body tissues. Late-stage infections progressed to the Malpighian tubules, gonads, and muscle tissues, in which spore loads were light to moderate. Measurements of fresh spores ($n = 50$) using an AEI Cook splitting image eyepiece were length 4.67 ± 0.41 μm (range 4.03–5.54 μm) and width 2.45 ± 0.25 μm (range 2.01–3.02 μm).

More than 90% of nymphs and adults and some eggs of *H. halys* from the DPI colony (Source 2) were found to be heavily infected with *N. maddoxi* when examined with light microscopy. Infections were systemic with particularly heavy infections in mid-gut and fat body tissues of nymphs and adults, and both vegetative stages and mature spores present (Fig. 1a–c). Dissected ovaries containing infected eggs which were porcelain white in appearance (Fig. 1d) and, upon examination, were found to be completely filled with spores.

Light micrographs and ultrastructural and molecular data determined that the microsporidium from Source 1, Source 2 and in all of the stink bug species examined from Pennsylvania (Source 3) were infected with *N. maddoxi* and had the following characteristics: Nuclei in all developmental stages were unpaired (unikaryotic, Fig. 2a–d, 3b). The earliest stages observed were unikaryotic late-stage meronts or early sporonts characterized by a ribosome-rich cytoplasm with few organelles (Fig. 2a). One sporulation sequence (sporogony plus spore morphogenesis) was observed. Proliferation of sporonts was by nuclear division without cytokinesis, and this produced multinucleate plasmodia with 4 or 8 nuclei (most common), but plasmodia with up to 16 nuclei were observed (Fig. 1a, 2b, c). Division of plasmodia occurred by plasmotomy to form smaller plasmodia (Fig. 1a) that divided into smaller unikaryotic sporonts by budding or forming elongate moniliform multinucleate stages that divided by multiple fissions. Late-stage sporonts became less dense and more elongate with prominent endoplasmic reticulum and a thickened plasmalemma (Fig. 2d).

Elongation and initiation of polar filament formation marked the transition from sporonts to sporoblasts (Fig. 2e). Early sporoblasts were often found in close association with host mitochondria and in some rare cuts were actually observed to fuse (Fig. 2e). As the sporoblast became more elongate, the plasma membrane thickened to initiate spore wall formation and genesis of the extrusion apparatus began in a highly synchronized and systematic process. Three distinct membrane-bound regions were formed in developing sporoblasts: one was involved in polar filament formation, one in the formation of the polaroplast, and one in the formation of the posterior vacuole (Fig. 3a). The posterior vacuole was an electron lucent region containing a rather sparse network of large tubules (Fig. 3a, b). Polar filament formation involved a centrally located region of Golgi bodies embedded in a dense membrane-bound matrix that appeared to extend to the anterior portions of the sporoblast, with this region presumably

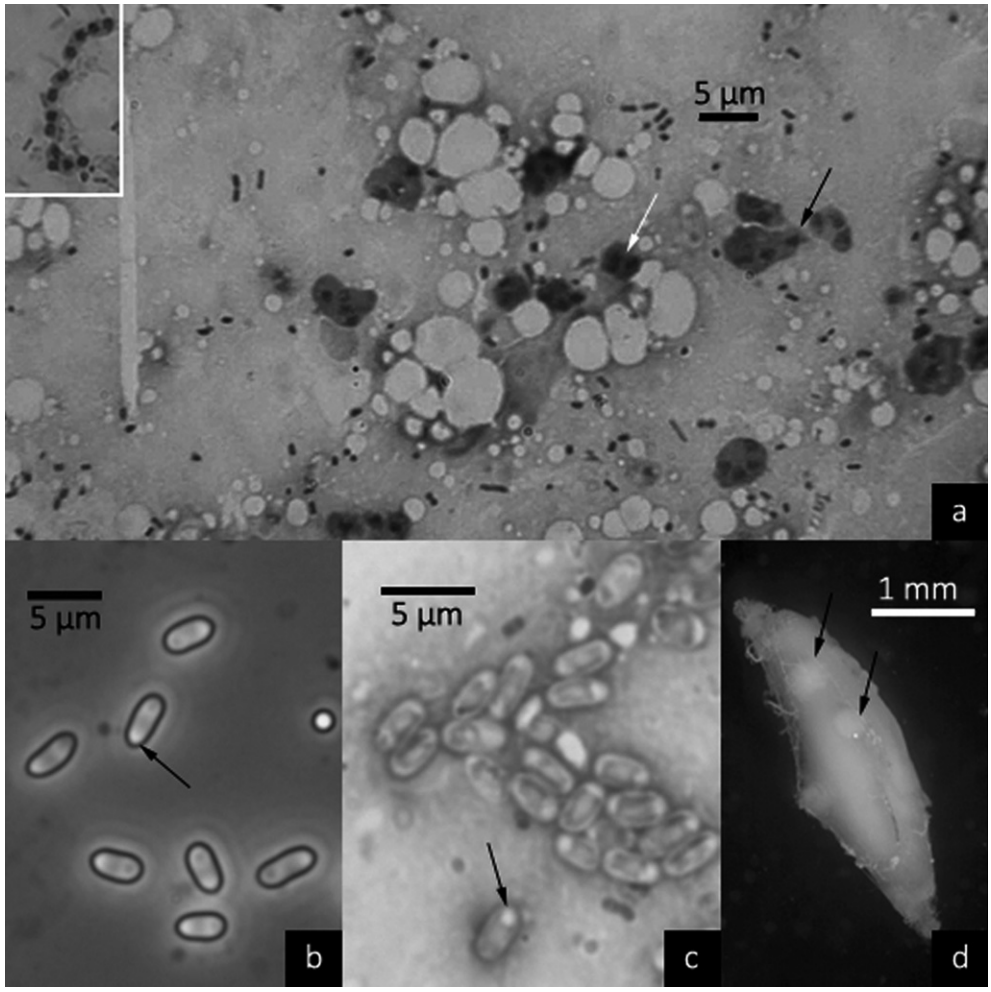

Figure 1 Developmental stages of *Nosema maddoxi* sp. nov. in adults of the brown marmorated stink bug *Halyomorpha halys* (Stål). (**a**) Binucleate (white arrow) and multinucleate stages of *N. maddoxi* in various stages of division. Note that all nuclei are unpaired. Black arrow indicates plasmodium undergoing plasmotomy. Dividing, moniliform plasmodia are shown in the inset; the top plasmodium is dividing into eight unikaryotic sporonts. (**b, c**) Groups of oblong spores with a prominent posterior vacuole (PV) at one pole (arrow). (**d**) Ovary dissected from an infected adult female containing infected eggs with a porcelain white coloration (arrow). a, c, Giemsa-stained; b, fresh, phase contrast.

involved in formation of the anchoring disc (Fig. 3a, 4a, b). Polaroplast formation began as a large, membrane-bound electron lucent region in the anterior region of the sporoblast, which initially contained a large network of medium-sized tubules and vacuoles (Fig. 3a, 4a). The tubules became filled with an electron-dense material and organized into a condensed concentric circle (Fig. 4b, c). As the spore matured, this region continued to expand in volume and formed a large concentric array of tubules that eventually flattened against one another to form the bipartite polaroplast in mature spores (Fig. 4d, 5b, c).

Spores were oblong with a distinct posterior vacuole and measured $4.72 \pm 0.05 \times 2.19 \pm 0.03$ µm (mean ± SE, fresh, $n = 30$) and $4.06 \pm 0.05 \times 2.07 \pm 2.07$ µm (mean ± SE, fixed, $n = 30$) (Fig. 1b, c), similar to measurements of *C. hilaris* spores (Source 1) using the image-splitting method. The polar filament (PF) was isofilar, making 7–9 turns around the posterior vacuole (Fig. 5a, d). The bipartite membranous polaroplast (PP) was composed of two distinct regions that occupied about a quarter of the anterior end of the spore (Fig. 5b, c). The anterior region of the polaroplast was composed of tightly packed lamellae, with a posterior region composed of loosely packed lamellae (Fig. 5c). The endospore began as a thick region in immature spores (Fig. 5c) that condensed into a thinner electron lucent region in mature spores (Fig. 5d). The exospore was thin and unlayered (Fig. 5c, d).

Sequence-based phylogenetic analyses

An alignment of microsporidian SSU sequences obtained from one *H. halys* (Source 2, DPI colony), one *H. halys* (Source 4, UMD colony), four *C. hilaris*, one *Euschistus tristigmus* (Say), and one *E. servus* from Pennsylvania (Source 3, all hosts confirmed by COI sequence data) showed 1,061/1,062 bp identity, indicating that they are

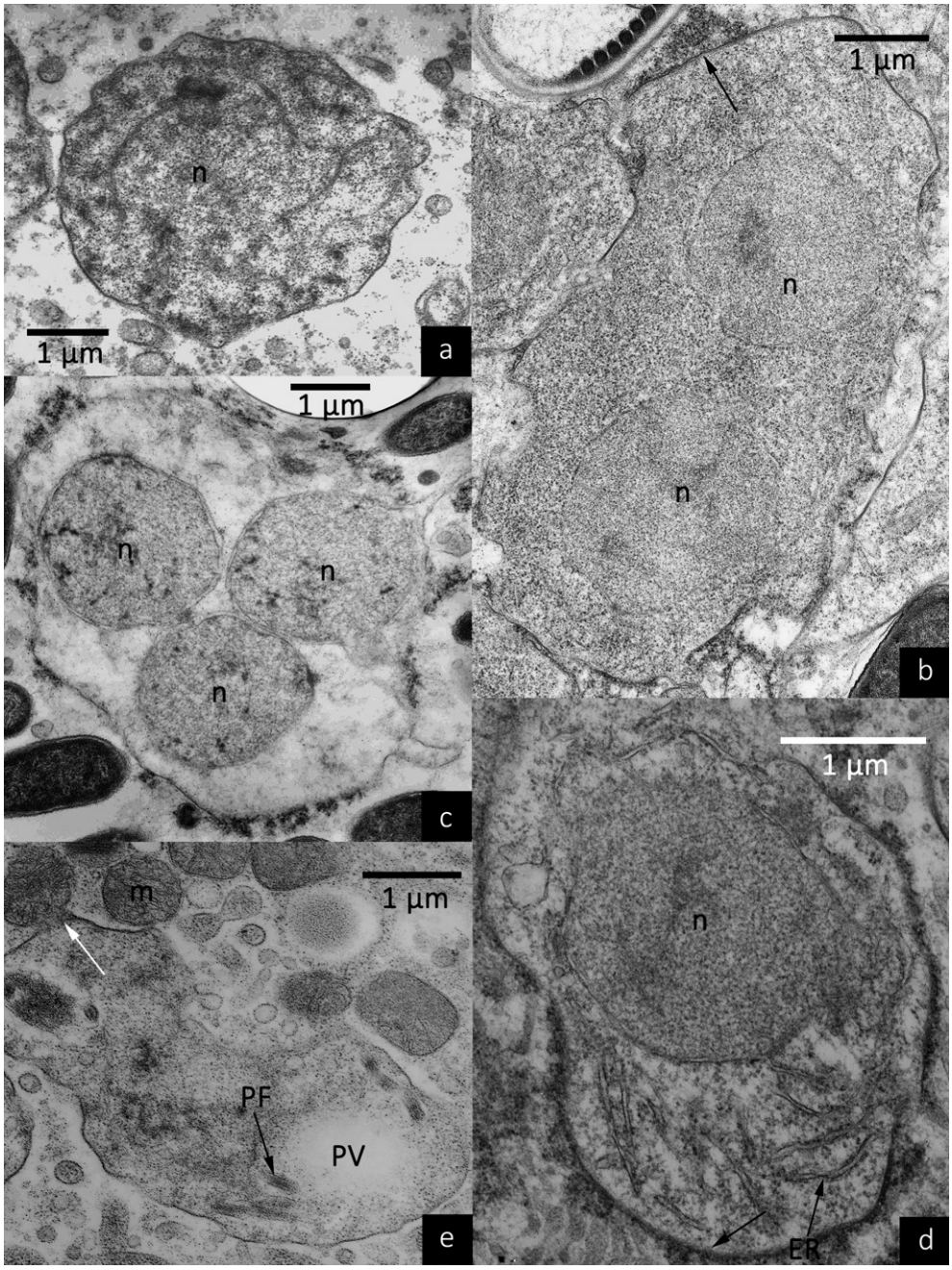

Figure 2 Transmission electron micrographs of developmental stages of *Nosema maddoxi* sp. nov. in adults of the brown marmorated stink bug *Halyomorpha halys* (Stål) and the green stinkbug *Chinavia hilaris* (Say). (**a**) Late-stage unikaryotic (n) meront. (**b**) Sporont with two nuclei (n) and a thickening plasmalemma (arrow). (**c**) Sporogonial plasmodium with three nuclei (n). (**d**) Late-stage unikaryotic sporont becoming elongate with a less dense cytoplasm, thickened plasmalemma (arrow) and an increase in endoplasmic reticulum (ER). (**e**) Early-elongate sporoblast with initiation of polar filament formation (PF) and posterior vacuole (PV). Note close association with host cell mitochondria (m), with one fused with the plasmalemma of the sporoblast (white arrow).

the same species, *N. maddoxi*. BLASTn analysis of this 1,062 bp sequence showed high per cent identity for members of the true *Nosema* clade represented by the type species *N. bombycis* (Table 3).

Assuming an SSU-ITS-LSU configuration, we attempted to amplify past the small subunit and into the interspacer and large subunit regions. This proved unsuccessful, and therefore, we used primers from Huang et al. (2004), which identified a reverse order of ribosomal subunits in *N. bombycis* (LSU-ITS-SSU). These primers (*LS228f* and *1537r*) produced a 4 kb amplicon, which, after cloning and sequencing, confirmed that this "reverse" ordering of

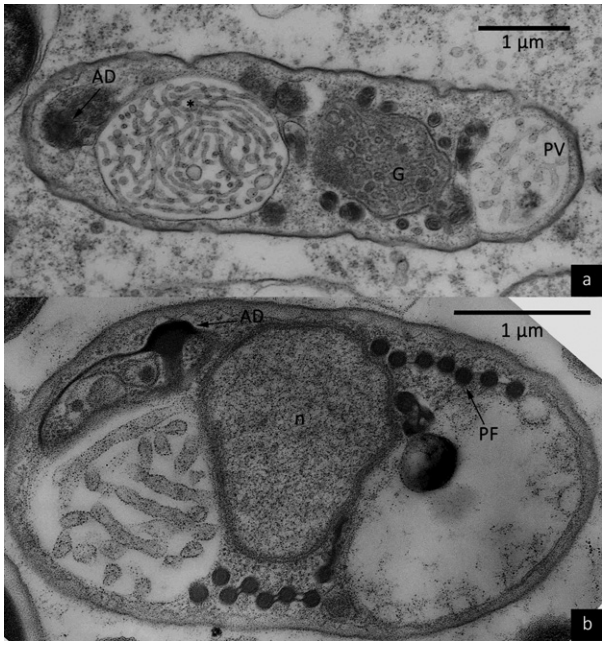

Figure 3 Transmission electron micrographs of sporoblasts and spore morphogenesis in *Nosema maddoxi* sp. nov. (**a**) Sporoblast demonstrating genesis of the anchoring disc (AD) and three distinct membrane-bound regions involved in polaroplast formation (*), polar filament formation involving the golgi bodies (G), and the posterior vacuole (PV). (**b**) Advanced stage sporoblast with a distinct anchoring disc (AD) and nearly complete polar filament formation (PF) and a centrally located nucleus (n).

subunits was present in the stink bug microsporidia (Fig. 6). Interestingly, the full-length product could not be sequenced directly because the ITS region proved highly variable, as Liu et al. (2013) reported for *N. bombycis* isolates from China. Those species that have the LSU-ITS-SSU configuration are indicated in Fig. 7.

By direct sequencing of amplicons generated using the universal primers, SSU sequences were obtained from two cryo-stored samples of the green stink bug *C. hilaris* infected with a microsporidium collected by Dr. Joe Maddox in 1968 (Source 1, Illinois). These sequences were 100% identical to the initial SSU consensus sequence described above. Through cloning, LSU-ITS-SSU sequences were also obtained from this material.

Thus, having sequence data from individual clones for the LSU-ITS-SSU region from the green stink bug *C. hilaris*, Source 1 (nine total clones from two individuals); the brown marmorated stink bug *H. halys*, Source 2 (nine clones from one individual); and the brown stink bug *E. servus,* Source 3 (seven clones from one individual), consensus sequences were created for the LSU and the SSU regions; the ITS region was too variable to define a consensus sequence (Fig. 6). The LSU consensus sequence was 2,393 bp, with a GC content of 30.1%. The SSU consensus sequence was 1,235 bp, with a GC content of 33.2%. The individual clones (containing the LSU-ITS-SSU region) used for assembling these consensus sequences were deposited under GenBank accession #s KY783594–KY783624.

One adult *H. halys* collected from South Korea was heavily infected, and a light infection was observed in one adult from Yunnan Prov., China. Sequencing of these two specimens with the SSU universal microsporidia primers *18f* and *1492r* resulted in amplicons of ~1,300 bp size. Based on these positive results, additional specimens were sequenced from the remaining Asian sites and all produced amplicons of ~1,300 bp size. Sequence data from nine samples showed < 6 bp difference from the SSU consensus sequence for *N. maddoxi* or approximately 99% identity.

Phylogenetic analysis of the consensus SSU region (Fig. 7) firmly places *N. maddoxi* in the "true *Nosema*" clade of the *Nosema/Vairimorpha* group (the Nosematidae). *Nosema maddoxi* had a 97.25% identity with the type species *N. bombycis*, 84% identity with the type species *V. necatrix* and between ~95% and 97% identity to other "true *Nosema*" species (Table 3). Within the "true *Nosema*" clade, *N. maddoxi* generally grouped with species from nonlepidopteran hosts (Fig. 7 and Table 2), including *Nosema empoascae* Ni, Backus, and Maddox from a hemipteran host. Other species in this group with insect hosts were *Nosema furnacalis* (Wen and Sun) from a lepidopteran and *Nosema chrysoperlae* Bjornson, Steele, Hu, Ellis, and Saito from a neuropteran. This group also included several species from noninsect hosts including *Nosema granulosis* Terry, Smith, Bouchon, Rigaud, Duncanson, Sharpe, & Dunn, 1999 from an amphipod and *Vairimorpha cheracis* Moodie, Le Jambre, & Katz, 2003 from a decapod (Terry et al.1999; Moodie et al. 2003). Sequence data for one other undescribed-related species isolated from a hemipteran host (*Nosema* sp. MC) did not group with the stink bug microsporidium but was also in the "true *Nosema*" clade (Fig. 7). An undescribed *Nosema* sp. from the hemipteran *L. lineolaris* (Table 1) grouped with the "true *Nosema*" clade (Musser et al. 2012), but as no morphological data are available for this species, it was not included in the phylogenetic analysis.

Infectivity and host specificity

In Bioassay 1, 60.0% of nymphs contained spores after death and all died as second instars 10 d after the egg mass was inoculated. In Bioassay 2, 72.2% of nymphs were infected, 50.0% died from infection at 21.5 d after egg mass inoculation (range: 6–35 d to death) and 84.6% of nymphs died as second instars. Only one control insect died within 40 d of hatching (3.8%), but it was not infected.

Laboratory host range testing of *N. maddoxi* from *C. hilaris* showed infectivity to both adult and nymphal *C. hilaris* and to some of the hemipteran species fed *N. maddoxi* spores, including the pentatomids *E. variolarius*, *P. maculiventris*, and both colour forms of *N. viridula,* as well as the lygaeid *O. fasciatus*. The mirid *L. lineolaris* and the larvae of the lepidopteran species *S. virginica*,

Figure 4 Transmission electron micrographs demonstrating polaroplast genesis in *Nosema maddoxi* sp. nov. (a–d) Polaroplast formation occurs within a large membrane-bound lucent region (*) in the anterior region of the sporoblast. Initially, the vacuole contains a large network of medium-sized tubules and vacuoles (a) that became filled with electron-dense material (b) and become organized into a condensed, concentric circle (b, c). This eventually evolved into a large, concentric array of tubules that eventually flattened against one another (d) and evolved into the bipartite lamellar polaroplast of the mature spore. PF = polar filament; AD = anchoring disc; G = golgi.

M. unipuncta, and *H. zea* were not susceptible. Examination of the parasitoid *T. japonicus* for the presence of spores was negative for all individuals. Molecular probes were not used on the parasitoids to detect possible cryptic infections but could be included in future studies to confirm they are not susceptible.

Field prevalence

Nosema maddoxi was isolated from field collections of *C. hilaris* in Monticello, IL, from 1970 to 1972 (Table 4). Infection prevalence ranged from 14.3% to 58.8% with no consistent trend in prevalence across seasons. In 1972,

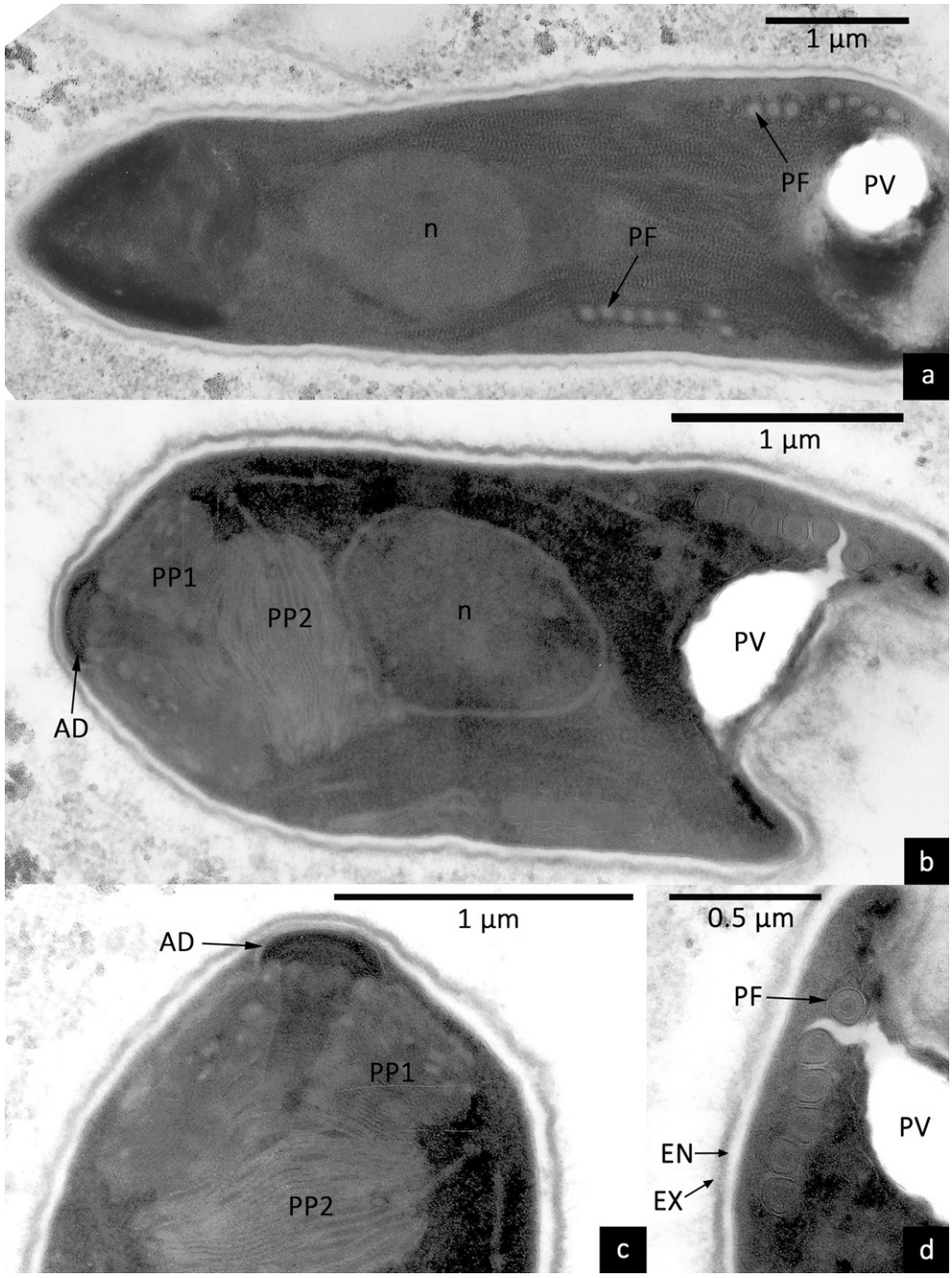

Figure 5 Ultrastructural features of the unikaryotic spores of *Nosema maddoxi* sp. nov. (**a**) Mature spore with seven coils of the polar filament (PF), prominent posterior vacuole (PV), and centrally located nucleus (n). (**b, c**) Mature spore with a centrally located nucleus (n), a typical anchoring disc (AD), and a bipartite polaroplast composed of an anterior region of tightly packed lamellae (PP1) and a posterior region of loosely packed lamellae (PP2). (**d**) Details of the polar filament (PF) in cross-section and the electron lucent endospore (EN), unlayered exospore (EX), and a collapsed posterior vacuole (PV).

overall prevalence of 29.7% in adult males (n = 128) was less than the overall prevalence in adult females (n = 93) of 52.7% (Fisher's exact test; P = 0.00078). Three other Illinois sites were monitored in 1971. Overall summer prevalences were 11.1% (N = 63) in Collinsville (Madison Co.), IL; 20.0% (N = 35) in Cobden (Union Co.), IL; and 8.3% (N = 12) in Princeville (Peoria Co.), IL. Prevalences of up to 50% were recorded on some individual dates. Infections that appeared morphologically identical to *N. maddoxi* were also found in field-collected *E. variolarius*, and *N. viridula*. One *N. maddoxi* isolate was also recovered from a *C. hilaris* adult in Florida during this time period.

Table 3. Per cent identity[a] of clade members to *Nosema maddoxi* and representative *Nosema* and *Vairimorpha* type species

Species	stink_bug	*N. bombycis*	*V. necatrix*
N. maddoxi	100.00	97.25	85.00
N. bombycis	97.25	100.00	84.32
N. heliothidis	97.82	99.43	84.69
N. spodopterae	97.82	99.43	84.69
N. trichoplusiae	97.82	99.35	84.70
N. tyriae	97.49	99.03	84.54
N. antheraeae	97.49	98.95	84.61
N. pyrausta	97.59	99.17	84.10
V. imperfecta	97.73	99.03	84.52
N. fumiferanae	97.72	99.24	84.82
V. ceraces	97.26	99.09	83.91
Endoreticulatus sp.	97.27	98.84	84.05
Microsporidium sp. ZYS-3	NA[b]	NA[b]	NA[b]
N. plutellae	96.69	98.22	83.92
N. disstriae	97.26	98.71	84.25
N. mylitta	NA[b]	NA[b]	NA[b]
N. granulosis	97.95	97.27	84.15
N. furnacalis	97.74	97.49	85.02
N. empoascae	97.24	96.99	83.84
N. chrysoperlae	95.18	93.42	84.36
N. sp._MC	97.65	99.27	84.77

[a]Per cent identity was calculated using the identity calculator based on a MUSCLE alignment of all clade members shown in Fig. 7.
[b]*N. mylitta* and *Microsporidium* sp. ZYS-3 are partial sequences and are excluded from the identity analysis.

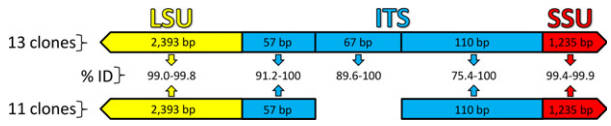

Figure 6 Gene architecture of *Nosema maddoxi* sp. nov. large ribosomal subunit (LSU), internal transcribed spacer (ITS), and small ribosomal subunit (SSU). Consensus sequences for LSU and SSU were created from multiple clones sequenced from the green stink bug *Chinavia hilaris* (eight clones), the brown marmorated stink bug *Halyomorpha halys* (nine clones), and brown native stink bug, *Euschistus servus* (seven clones). The ITS region showed too much variability to generate a consensus, and 13/24 clones showed 67 extra internal bp. Representative lengths are shown, as well as % ID range to a single clone.

Field collections of *H. halys* from five eastern U.S. states during 2015 and 2016 showed variability in prevalence. *Nosema maddoxi* infections were found in four of the five states and in 11 of the 18 collections (Table 5). For these collections, infection prevalence in *H. halys* ranged from 0.0% to 28.3%. In collections of native stink bugs in the subfamily Pentatominae that were sympatric with *H. halys,* only *C. hilaris* were found to be infected, with prevalences of 29.4 ($n = 34$) and 33.3% ($n = 27$) in Biglerville and Fairfield, PA, respectively. For these collections, prevalence of *N. maddoxi* in *C. hilaris* was not significantly different from its prevalence in *H. halys*

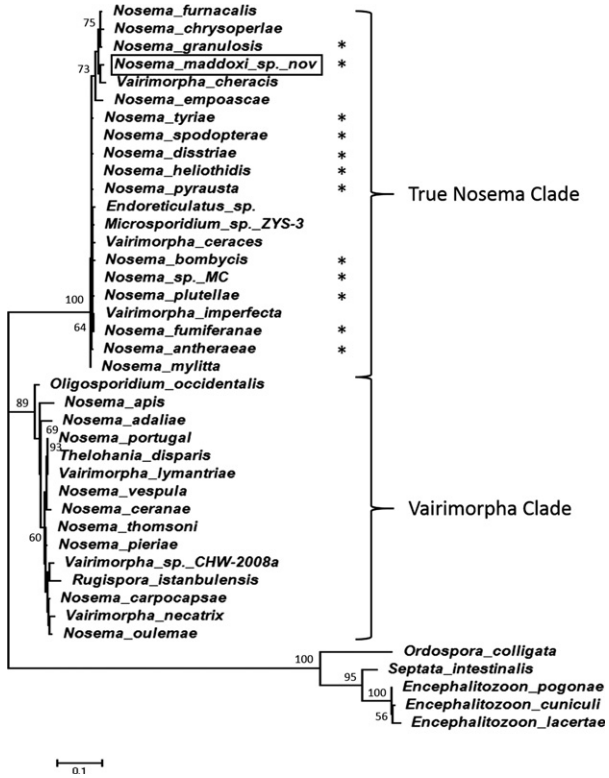

Figure 7 Maximum-likelihood phylogeny of selected ribosomal small subunit sequences that places *Nosema maddoxi* sp. nov. with the *Nosema bombycis* ("true *Nosema*) clade of microsporidia. Species marked with an asterisk are confirmed to have the LSU-ITS-SSU configuration of the ribosomal gene.

collected at the same sites and times (Fisher's exact tests; $P > 0.05$).

DISCUSSION

The developmental and ultrastructural features for a novel microsporidium from the green stink bug *C. hilaris* (collected in Illinois and characterized by Maddox in 1979, and named here *Nosema maddoxi* sp. nov.) match the morphological features of samples collected recently from *H. halys* and *C. hilaris* in the mid-Atlantic region. In addition, there is 100% SSU sequence identity between *N. maddoxi* collected recently and the voucher specimens preserved by Maddox, confirming the presence of *N. maddoxi* in the midwestern U.S. prior to the introduction of *H. halys* to the mid-Atlantic U.S. Our study has also confirmed the widespread presence of *N. maddoxi* in both China and South Korean populations *of H. halys*. These findings therefore suggest that *N. maddoxi* had a Holarctic distribution before the introduction of *H. halys* to North America. Other studies have suggested that some microsporidian species have Holarctic distributions, for example *Nosema bombi* in bumble bees (Solter 2014), and the *N. bombycis*-type isolates from noctuid Lepidoptera (e.g. *Nosema disstriae* and *Nosema trichoplusiae;* Vossbrinck and Debrunner-Vossbrinck 2005).

Table 4. Prevalence of *Nosema maddoxi* infection in *Chinavia hilaris* from summer light-trap collections in Monticello (Piatt County), Illinois

Month and year	% Infected (*N*)
1970	
July	48.5 (99)
August	24.3 (70)
1971	
June	20.0 (5)
July	28.6 (7)
August	14.3 (7)
September	33.3 (15)
October	33.3 (12)
1972	
May	18.2 (11)
June	16.9 (83)
July	58.8 (68)
August	51.5 (33)
September	50.0 (26)

N = total *C. hilaris* collected.

Invasive species can arrive at new locations accompanied by coevolved pathogens that may easily go undetected. For example, strains of the nematode *Deladenus siricidicola* Bedding accompanied the Eurasian woodwasp, *Sirex noctilio* F. when it was introduced to New Zealand (Zondag 1969, 1975) and to North America (Yu et al. 2009). Alternatively, invasive species may be susceptible to pathogens and parasites that are endemic in native (often related) host species. When the soya bean aphid, *Aphis glycines* Matsumura, was introduced to North America, a diversity of North American native fungal pathogens began infecting the invader (Nielsen and Hajek 2005). However, it is not always a simple matter to determine whether a pathogen attacking a newly invasive species was introduced with the host or is endemic to the invaded area. In particular, when invasive species are not major pests in their areas of origin, associated pathogens in the area of endemism might not be known. In the present study, the existence of *N. maddoxi* in Asia where *H. halys* is native was not known prior to our collections and the infection of brown marmorated stink bugs by *N. maddoxi* in North America had not previously been reported. Nevertheless, whether *H. halys* carried *N. maddoxi* to North America when it was introduced is not known because the same species was already present in native stink bugs, especially the green stink bug, *C. hilaris*, (Maddox 1979), in North America.

We have detected *N. maddoxi* throughout the eastern mid-Atlantic states in the U.S. in our distributional studies and, because it is apparently native to the U.S. (known from stink bugs in Illinois and Florida in 1968–1972), we hypothesize that *N. maddoxi* may be found throughout the distribution of established *H. halys* populations. It may already be present in native stink bug populations but could also be introduced or re-introduced along with *H. halys*. In support of this theory, we performed a BLASTn search on transcriptome RNAseq data from a *H. halys* colony established from specimens collected in Allentown, PA (BioProject accession # PRJNA242849; Sparks et al. 2014) and found reads matching 100% identity across the length of the *N. maddoxi* SSU sequence. Good quality control and disease management strategies

Table 5. Infection of *Halyomorpha halys* with *Nosema maddoxi* in the eastern United States in 2015–2016

State	County	Location	GPS	Collection date	#Diagnosed	#Infected	%Infection
2015							
New York	Tompkins	Ithaca	42°26′35.6″N, 76°27′39.4″W	6–15 October	31	0	0.0%
Maryland	Prince George's	Beltsville	39°01′25.608″N, 76°56′1.889″W	17-September	17	0	0.0%
Pennsylvania	Lancaster	Lancaster	39°53′29″N, 76°45′51″W	11-September	50	0	0.0%
Pennsylvania	Adams	Mont Tabor	40°0′34″N, 77°14′15″W	11-September	192	40	20.8%
Pennsylvania	Adams	Aspers	39°48′59″N, 78°1′28″W	11-September	70	10	14.3%
Pennsylvania	Adams	Biglerville	39°57′2″N, 77°18′38″W	11-September	22	2	9.1%
Virginia	Giles	Pearlsburg	37°19′46.0992″N, 80°42′57.9744″W	20-September	42	4	9.5%
Virginia	Montgomery	Blacksburg	37°13′48″N, 80°25′04.4″W	6-October	59	4	6.8%
Virginia	Montgomery	McCoy	37°12′6.0012″N, 80°33′50.5836″W	21-September	19	2	10.5%
2016							
New York	Wayne	Williamson	43°14′21.10″N, 77°10′17.48″W	16-September	85	3	3.5%
New York	Ontario	Farmington	42°56′41.72″N, 77°20′07.61″W	10-September	5	0	0.0%
New York	Tompkins	Ithaca	42°26′37″N, 76°30′02″W	10-September to 17-October	12	0	0.0%
New York	Ulster	Highland	41°44′46.07″N, 73°57′52.85″W	14-September	68	0	0.0%
Pennsylvania	Indiana	Hillsdale	40°45′05.77″N, 78°53′01.13″W	7-October	4	0	0.0%
Pennsylvania	Lancaster	Lancaster	39°97′71″N, 76°30′74″W	20-September	149	1	0.7%
Pennsylvania	Adams	Biglerville	39°95′67″N, 77°30′96″W	20-September	331	40	12.1%
Pennsylvania	Adams	Fairfield	39°81′37″N, 77°39′06″W	20-September	99	28	28.3%
North Carolina	Henderson	Mills River	35°42′88.79″N, 82°56′49.74″W	13-August, 8–19-September	130	22	16.9%

must be used when establishing laboratory colonies of stink bugs to ensure they are free of this often chronic but deleterious pathogen.

Phylogenetic analysis of the consensus SSU region of the ribosomal gene places *N. maddoxi* in the "true *Nosema*" clade of microsporidia, with *N. bombycis* as the type species (Fig. 7). The genus *Nosema* has long been recognized to contain many heterogeneous species based on morphological and biological features prior to the availability of molecular data (see comprehensive review by Kyei-Poku and Sokolova 2017). This study and previous studies using SSU DNA phylogenies have indicated that there are two subgroups within the "true *Nosema*" clade with one of these mostly from non-Lepidopteran hosts (Kyei-Poku and Sokolova 2017). The nonmolecular features for the "true *Nosema*" clade have been revised over time with the general characteristics being diplokaryotic developmental stages, diplokaryotic spores, all stages in direct contact with the host cell cytoplasm and disporoblastic sporogony (Canning and Lom 1986; Kyei-Poku and Sokolova 2017; Larsson 1988; Sprague 1978; Sprague et al. 1992). Molecular data have resulted in the transfer of many species previously considered to belong to *Nosema* based on morphological characters to other genera. Well-known examples include *Nosema algerae* Vavra and Undeen transferred to *Anncaliia* and *Nosema locustae* Canning transferred to *Paranosema* (Franzen et al. 2006; Sokolova et al. 2003).

The reversed arrangement of the ribosomal gene (LSU–ITS–SSU) was first identified in *N. bombycis* (Huang et al. 2004) and has been confirmed in at least nine additional species within the "true *Nosema*" clade but not for any species within the "*Vairimorpha*" clade (Fig. 7). It has been suggested that the reversed arrangement of the ribosomal gene may represent an important characteristic for "true *Nosema*" species (Kyei-Poku and Sokolova 2017; Tsai et al. 2005). This type of ribosomal architecture is not unique to the "true *Nosema*" clade having been identified in the unrelated species *Glugoides intestinalis* (Chatton) (Refardt and Mouton 2007) and a putative *Endoreticulatus* sp. (Xu et al. 2011). Factors that have resulted in this reversed arrangement of the ribosomal gene in some species of microsporidia are unclear (Xu et al. 2011), and it is not known how widespread this gene rearrangement is for the microsporidia. Relevant to this study, the presence of the reversed arrangement of the ribosomal gene, combined with similarities of the SSU sequence, represent important characteristics for species in the "true *Nosema*" clade that distinguish it from the "*Vairimorpha*" clade (Kyei-Poku and Sokolova 2017).

Franzen (2008) stated that "microsporidian taxonomy has obviously reached a breaking point where classical morphological-based methods are losing importance and will be supplemented or even replaced by molecular-based methods." He further concluded that several morphological characters (number of nuclei and other details of the life cycle) may change very rapidly during adaptation to different hosts, and tissues and some characters seem to have evolved several times simultaneously. *Nosema maddoxi* is a case in point; developmental stages and spores are unikaryotic, but molecular data place it with the "true *Nosema*" clade where the primary morphological characters are diplokaryotic vegetative stages and spores. This study and a recent report using RPB1 analysis found that the species from nonlepidopteran hosts formed a subgroup within the true *Nosema* clade (Kyei-Poku and Sokolova 2017). *Nosema maddoxi* falls within this group that includes *N. empoascae*, *V. cheracis,* and *N. granulosis*. The exception in this subgroup is *N. furnacalis* which is from a lepidopteran host.

While the molecular data and morphological characters are in agreement for the majority of the species within the "true *Nosema*" clade, there are species with conflicting morphological features such as *N. empoascae*, *N. granulosis*, *V. cheracis*, *V. imperfecta,* and an *Endoreticulatus* sp. *Nosema empoascae* (from a hemipteran host), has both unikaryotic and diplokaryotic developmental stages but only diplokaryotic spores (Ni et al. 1995), and unikaryotic stages also have been reported for *N. bombycis* by various authors (see Sprague et al. 1992 for a review). *Nosema granulosis* (from a crustacean host) has only diplokaryotic stages and spores that have an unusual granular polaroplast but is only known to be transovarially transmitted (Terry et al. 1999). *Vairimorpha cheracis* (from a crustacean host) has one sequence that produces diplokaryotic spores but also has a meiotic sequence (along with other species in the genus *Vairimorpha* and abortive in *V. imperfecta*) that produces unikaryotic spores (Canning et al. 1999; Moodie et al. 2003; Pilley 1976). There is also an undescribed *Endoreticulatus* sp. in this clade that presumably has only unikaryotic stages and spores (Wang et al. 2001; genus designation unsubstantiated due to lack of morphological data). Taken together with the description of *N. maddoxi*, the number of nuclei in developmental stages and spores can vary for members of the true *Nosema* clade as well as for other genera within the *Nosema/Vairimorpha* group such as *Oligosporidim* and *Rugispora* (Fig. 7) that are unikaryotic throughout development (Becnel et al. 2002; Bekircan et al. 2017).

Kyei-Poku and Sokolova (2017) recognized two subgroups within the true *Nosema* clade but did not transfer the members of this group to a new genus. *Nosema maddoxi* is another member of this subgroup, but we have concluded that creation of a new genus is not justified at this time because of the relatively small number of species within this subgroup and considerable variability in hosts, developmental sequences, and morphology. With information from this and previous studies referenced above, important characteristic features for most members of the genus *Nosema* are (i) similarities based on SSU sequence of the ribosomal gene; (ii) gene architecture of the ribosomal gene usually LSU–ITS–SSU; (iii) hosts are usually invertebrates, primarily insects; and (iv) sporogony is typically disporoblastic, and development is in direct contact with the host cell cytoplasm. The meiotic sequence in *V. cheracis* is in conflict with these developmental features, but it is known that meiotic sequences can be abortive or lost for some genera and species in

some clades, such as found for *V. imperfecta* that falls in the "true *Nosema*" clade (Canning et al. 1999) and in several genera within the *Amblyospora* clade in mosquitoes (Andreadis 2007; Becnel 1994).

Considering the available information, we propose that the morphological description for the genus *Nosema* be amended to state that developmental stages and spores are usually diplokaryotic in Lepidopteran host and variable in non-Lepidopteran hosts and that molecular features include similarities of the SSU sequence and usually the presence of the reversed arrangement of the ribosomal gene. These changes would provide flexibility when sequence data combined with ribosomal gene architecture are similar to the "true *Nosema*" clade and the number of nuclei in stages conflict with the classical characteristics for this genus. This is clearly the situation for *N. maddoxi* based on the morphological information presented in this description together with the molecular data that distinguishes it from all other described species. The authors responsible for *Nosema maddoxi* sp. nov. are Becnel, Solter, Hajek, Huang, Sanscrainte, and Estep.

TAXONOMIC SUMMARY

Phylum: Microsporidia Balbani 1882
Family: Nosematidae Labbé 1899
Genus: *Nosema* Naegeli 1857

Nosema maddoxi sp. nov. Becnel, Solter, Hajek, Huang, Sanscrainte, Estep

Synonymy: *Microsporidium* sp. Maddox, 1979; *Nosema* sp. Sparks et al., 2014.

Diagnosis

Type host: The green stink bug *Chinavia hilaris* (Say) (Hemiptera: Pentatomidae)

Host range: Naturally infected species included the brown marmorated stink bug, *Halyomorpha halys* (Stål); the brown stink bug, *Euschistus servus* (Say); and the dusky stink bug, *Euschistus tristigmus* (Say). Laboratory transmission to *Euschistus variolarius* (Palisot de Beauvois), *Podisus maculiventris* (Say), *Nezara viridula* (Linnaeus), and *Oncopeltus fasciatus* (Dallas) was successful, while *Lygus lineolaris* (Palisot de Beauvois), *Spilosoma (Diacrisia) virginica* (Fab.), *Mythimna (Pseudaletia) unipuncta* (Haworth), and *Helicoverpa (Heliothus) zea* (Boddie) were not susceptible. The egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), reared on infected *H. halys* (Source 2), was not infected.

Site of Infection: Systemic, but infection levels highest in mid-gut and fat body of immatures and adults. Eggs from infected females of *H. halys* contained mature spores.

Transmission: Horizontal transmission per os; transovum/transovarial transmission indicated.

Development: Nuclei in all developmental stages observed were unpaired (unikaryotic) and in direct contact with the host cell cytoplasm. Proliferation of sporonts was by nuclear division without cytokinesis that produced multinucleate plasmodia with 4 or 8 nuclei and occasionally 16 nuclei. Division of plasmodia occurred by plasmotomy to form smaller plasmodia that divided into unikaryotic sporonts by budding or forming elongate moniliform multinucleate stages that divide by multiple fissions.

Spore morphogenesis: Extrusion apparatus formation was highly synchronized and systematic. Three distinct membrane-bound regions were formed in developing sporoblasts, one involved in polar filament formation, one in formation of the polaroplast, and one in formation of the posterior vacuole.

Spores: Unikaryotic spores were oblong and measured $4.72 \pm 0.05 \times 2.19 \pm 0.03$ μm (mean ± SE, fresh, $n = 30$) and $4.06 \pm 0.05 \times 2.07 \pm 2.07$ μm (mean ± SE, fixed, $n = 30$). Spores contained a distinct posterior vacuole with 7–9 isofilar polar filament coils. A polaroplast composed of two distinct regions occupied about a quarter of the anterior end of the spore. The anterior region was composed of tightly packed lamellae, and the posterior region was composed of loosely packed lamellae. The spore wall was composed of an electron lucent endospore approximately twice the thickness of the electron-dense unlayered exospore.

Interface: All stages were in direct contact with the host cell cytoplasm.

Type locality: Monticello, Illinois, USA, for the *Chinavia hilaris* isolate.

Etymology: Named after Dr. Joseph Maddox who first discovered this microsporidium in *Chinavia hilaris*.

Molecular characterization: Gene architecture of ribosomal gene is LSU–ITS–SSU. The placement of *Nosema maddoxi*, based on phylogenetic analysis of the consensus SSU region, is within the "true *Nosema*" group of the *Nosema/Vairimorpha* clade**.** Deposits to GenBank were clones containing the LSU–ITS–SSU region from individual *H. halys*, *C. hilaris*, and *E. servus* (GenBank accession #s KY783594–KY783624).

Deposition of type specimens: Four type slides have been deposited with the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, DC (USNM Nos. 1422424–1422427). Additional slides and specimens embedded in plastic resin are archived at the Center for Medical, Agricultural and Veterinary Entomology, USDA/ARS, Gainesville, Florida.

ZooBank: http://zoobank.org/urn:lsid:zoobank.org:act:A0582FBF-3BFC-4C73-9370-72055BF186A0

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LITERATURE CITED

Andreadis, T. G. 2007. Microsporidian parasites of mosquitoes. *J. Am. Mosq. Control Assoc.*, 23:3–29.

Becnel, J. 1994. Life cycles and host-parasite relationships of Microsporidia in culicine mosquitoes. *Folia Parasitol.*, 41:91–96.

Becnel, J. J. 2012. Complementary techniques: preparations of entomopathogens and diseased specimens for more detailed study using microscopy. *In:* Lacey, L. A. (ed.), Manual of Techniques in Invertebrate Pathology. Academic Press, New York, NY. p. 337–353.

Becnel, J. J. & Andreadis, T. G. 2014. Microsporidia in insects. *In:* Weiss, L. M. & Becnel, J. J. (ed.), Microsporidia: Pathogens of Opportunity. John Wiley and Sons, Inc., Ames, IA. p. 521–570.

Becnel, J. J., Jeyaprakash, A., Hoy, M. A. & Shapiro, A. 2002. Morphological and molecular characterization of a new microsporidian species from the predatory mite *Metaseiulus occidentalis* (Nesbitt) (Acari, Phytoseiidae). *J. Invertebr. Pathol.*, 79:163–172.

Bekircan, C., Bülbül, U., Güler, H. & Becnel, J. J. 2017. Description and phylogeny of a new microsporidium from the elm leaf beetle, *Xanthogaleruca luteola* Muller, 1766 (Coleoptera: Chrysomelidae). *Parasitol. Res.*, 116:773–780.

Canning, E. U., Curry, A., Cheney, S., Lafranchi-Tristem, N. J. & Haque, M. A. 1999. *Vairimorpha imperfecta* n. sp., a microsporidian exhibiting an abortive octosporous sporogony in *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). *Parasitology*, 119:273–286.

Canning, E. U. & Lom, J. 1986. The microsporidia of vertebrates. Academic Press, London. p. 289.

Christophers, S. R. 1922. Editorial note on bodies found by Mrs. Adie in salivary glands of bed bug (Kala-Azar inquiry of the Indian Research Fund Association). *Indian J. Med. Res.*, 9(4):v.

Diarra, K. & Toguebaye, B. S. 1995. Ultrastructural study of *Nosema omaniae* sp. n. (Microspora, Nosematidae) parasite of *Omania coleoptrata* (Heteroptera, Omaniidae). *Acta Protozool.*, 34:61–66.

Fantham, H. B. & Porter, A. 1958. Some pathogenic bacteriform Microsporidia from Crustacea and Insecta. *Proc. Zool. Soc. Lond.*, 130:153–168.

Franzen, C. 2008. Microsporidia: a review of 150 years of research. *Open Parasitol. J.*, 2:1–34.

Franzen, C., Nassonova, E. S., Scholmerich, J. & Issi, I. V. 2006. Transfer of the members of the genus *Brachiola* (microsporidia) to the genus *Anncaliia* based on ultrastructural and molecular data. *J. Eukaryot. Microbiol.*, 53:26–35.

Galli-Valerio, B. 1924. Parasitologische untersuchungen und beitrage zur parositologischen technik. *Zentralbl. Bakteriol. Jena Abt I*, 91:120–125.

Ghosh, K. & Weiss, L. M. 2009. Molecular diagnostic tests for Microsporidia. *Interdiscip. Perspect. Infect. Dis.*, 2009:1–13.

Gouy, M., Guindon, S. & Gascuel, O. 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.*, 27:221–224.

Haye, T., Gariepy, T., Hoelmer, K., Rossi, J.-P., Streito, J.-C., Tassus, X. & Desneux, N. 2015. Range expansion of the invasive brown marmorated stink bug, *Halyomorpha halys*: an increasing threat to field, fruit and vegetable crops worldwide. *J. Pest. Sci.*, 88:665–673. https://doi.org/10.1007/s10340-015-0670-2.

Herlihy, M., Erwin, N. & Weber, D. 2014. Marmorating: A day in the life of the BMSB colony at USDA ARS Beltsville. Brown marmorated stink bug working group meeting, Winchester, Virginia, 3 Dec. http://www.ars.usda.gov/SP2UserFiles/person/30842/Herlihy-Erwin-Weber-BMSB-rearing-3Dec2014.pdf. Accessed 23 March 2017.

Hoebeke, E. R. & Carter, M. E. 2003. *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae): a polyphagous plant pest from Asia newly detected in North America. *Proc. Entomol. Soc. Wash.*, 105:225–237.

Huang, W.-F., Tsai, S.-J., Lo, C.-F., Soichi, Y. & Wang, C.-H. 2004. The novel organization and complete sequence of the ribosomal RNA gene of *Nosema bombycis*. *Fungal Genet. Biol.*, 41:473–481.

Kramer, J. P. 1972. *Octosporea carloschagasi* n. sp., a microsporidian associate of *Trypanosoma cruzi* in *Panstrongylus megistus*. *Z. Parasitenkd.*, 39:221–224.

Kumar, S., Stecher, G. & Tamura, K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.*, 33:1870–1874.

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.

Larsson, R. 1988. Identification of microsporidian genera (Protozoa, Microspora). A guide with comments on the taxonomy. *Arch. Protistenkd.*, 136:1–37.

Lee, D.-H., Short, B. D., Joseph, S. V., Bergh, J. C. & Leskey, T. C. 2013. Review of the biology, ecology, and management of *Halyomorpha halys* (Hemiptera: Pentatomidae) in China, Japan, and the Republic of Korea. *Environ. Entomol.*, 42:627–641.

Lipa, J. J. 1966a. Miscellaneous observations on protozoan infections of *Nepa cinerea* Linnaeus including descriptions of two previously unknown species of Microsporidia, *Nosema bialoviesianae* sp. n. and *Thelohania nepae* sp. n. *J. Invertebr. Pathol.*, 8:158–166.

Lipa, J. J. 1966b. *Nosema leptocoridis* sp. n., microsporidan parasite of *Leptocoris trivittatus* (Say) (Hemiptera). *J. Invertebr. Pathol.*, 8:250–251.

Lipa, J. J. 1977. *Nosema pyrrhocoridis* n. sp., a new microsporidian parasite of red soldier bug (*Pyrrhocoris apterus* L.) (Heteroptera, Pyrrhocoridae). *Acta Protozool.*, 16:135–140.

Lipa, J. J., Hazard, E. I. & Oldacre, S. W. 1975. *Chapmanium nepae*. *Tech. Bull. U.S. Dep. Agric.*, 1530, p. 48.

Liu, H. J. & McEwen, F. L. 1977. *Nosema blissi* sp. n. (Microsporida: Nosematidae), a pathogen of the chinch bug, *Blissus leucopterus hirtus* (Hemiptera: Lygaeidae). *J. Invertebr. Pathol.*, 29:141–146.

Liu, H., Pan., G., Luo, B., Ti, L., Yang, Q., Vossbrinck, C. R., Debrunner-Vossbrinck, B. A. & Zhou, Z. 2013. Intraspecific polymorphism of rDNA among five *Nosema bombycis* isolates from different geographic regions in China. *J. Invertebr. Pathol.*, 113:63–69.

Maddox, J. V. 1979. Ultrastructural observations on the development of a microsporidium of the green stink bug *Acrosternum hilare*. *Proc. Internatl. Colloq. Invertebr. Pathol. & Ann. Mtg. Soc. Invertebr. Pathol.*, Sept. 11–17, 1978, Prague, pp. 121–122.

Milano, P., Cônsoli, F. L., Zério', N. G. & Parra, J. R. P. 1999. Thermal requirements of the cotton stainer *Dysdercus peruvianus* Guerin-Meneville (Heteroptera: Pyrrhocoridae). *An. Soc. Entomol. Bras.*, 28:233–238.

Moodie, E. G., Le Jambre, L. F. & Katz, M. E. 2003. Ultrastructural characteristics and small subunit ribosomal DNA sequence of *Vairimorpha cheracis* sp. nov. (Microspora: Burenellidae), a parasite of the Australian yabby, *Cherax destructor* (Decapoda: Parastacidae). *J. Invertebr. Pathol.*, 84:198–213.

Musser, F. R., Knighten, K. S. & Self, S. R. 2012. Impact of *Nosema* (Microsporidia) infections and fumagillin treatment on

Lygus lineolaris (Hemiptera: Miridae). *J. Invertebr. Pathol.*, 111:101–105.
Ni, X., Backus, E. A. & Maddox, J. V. 1995. A new Microsporidium, *Nosema empoascae* n. sp., from *Empoasca fabae* (Harris) (Homoptera: Auchenorrhyncha: Cicadellidae). *J. Invertebr. Pathol.*, 66:52–59.
Nielsen, C. & Hajek, A. E. 2005. Control of invasive soybean aphid, *Aphis glycines* (Hemiptera: Aphididae) populations by existing natural enemies in New York State. *Environ. Entomol.*, 34:1036–1047.
Northeastern IPM Center. 2016. Stop BMSB. Cornell University, Ithaca, NY. http://www.stopbmsb.org/. Accessed 31 March 2017.
Pilley, B. M. 1976. A new genus, *Vairimorpha* (Protozoa: Microsporida), for *Nosema necatrix* Kramer 1965: pathogenicity and life cycle in *Spodoptera exempta* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol.*, 28:177–183.
Poisson, R. 1924. Sur quelques Microsporidies parasites d' Arthropodes. *C.R. Acad. Sci.*, 178:664–666.
Poisson, R. 1928. Sur une infection à microsporidie chez la nèpe cendrées (Hémipetere-Héteroptère) la réaction des tissues l'hôte vis-à-vis du parasite. *Arch. Zool. Exp. Gén.*, 67:129–137.
Poisson, R. 1929. Recherches sur les microsporidies parasite des hémipteres. *Arch. Zool. Exp. Gén.*, 69:55–63.
Poisson, R. 1941. Les microsporidies parasites des insects hémiptères. IV. Sur une microsporidie: *Toxoglugea gerridis,* nov. spec. d' *Aquarius najas* de Ger (Gerridae). *Arch. Zool. Exp. Gén.*, 82:30–35.
Refardt, D. & Mouton, L. 2007. Reverse arrangement of rRNA subunits in the microsporidium *Glugoides intestinalis*. *J. Eukaryot. Microbiol.*, 54:83–85.
Shortt, H. E. & Swaminath, C. S. 1924. A note on *Nosema adiei*. *Indian J. Med. Res.*, 12:181–183.
Sokolova, Y. Y., Dolgikh, V. V., Morzhina, E. V., Nassonova, E. S., Issi, I. V., Terry, R. S., Ironside, J. E., Smith, J. E. & Vossbrinck, C. R. 2003. Establishment of the new genus *Paranosema* based on the ultrastructure and molecular phylogeny of the type species *Paranosema grylli* gen. nov., comb. nov. (Sokolova, Selezniov, Dolgikh, Issi 1994), from the cricket *Gryllus bimaculatus* Deg. *J. Invertebr. Pathol.*, 84:159–172.
Solter, L. F. 2014. Epizootiology of invertebrate Microsporidia. *In:* Weiss, L. M. & Becnel, J. J. (ed.), Microsporidia: Pathogens of Opportunity. Wiley-Blackwell, Ames, IA. p. 165–194.
Sparks, M. E., Shelby, K. S., Kuhar, D. & Gunderson-Rindal, D. E. 2014. Transcriptome of the invasive brown marmorated stink bug, *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae). *PLoS ONE*, 9(11):e111646.
Sprague, V. 1977. Annotated list of species of Microsporidia. *In:* Bulla, L. A. & Cheng, T. C. (ed.), Comparative Pathobiology. Plenum Press, New York, NY. p. 31–334.
Sprague, V. 1978. Characterization and composition of the genus *Nosema*. *Misc. Publ. Entomol. Soc. Am.*, 11:5–16.
Sprague, V., Becnel, J. J. & Hazard, E. I. 1992. Taxonomy of phylum Microspora. *Crit. Rev. Microbiol.*, 18:285–395.
Terry, R. S., Smith, J. E., Bouchon, D., Rigaud, T., Duncanson, P., Sharpe, R. G. & Dunn, A. M. 1999. Ultrastructural characterisation and molecular taxonomic identification of *Nosema granulosis* n. sp., a transovarially transmitted feminising (TTF) microsporidium. *J. Eukaryot. Microbiol.*, 46:492–499.
Tonka, T. & Weiser, J. 2000. *Becnelia sigarae* gen. n., sp. n. isolated from testes of the water boatmen, *Sigara lateralis* (Heteroptera: Corixidae) in the Czech Republic. *Acta Protozool.*, 39:241–252.
Tsai, S. J., Huang, W. F. & Wang, C. H. 2005. Complete sequence and gene organization of the *Nosema spodopterae* rRNA gene. *J. Eukaryot. Microbiol.*, 52:52–54.
Vavra, J., Hylis, M., Vossbrinck, C. R., Pilarska, D. K., Linde, A., Weiser, J., McManus, M. L., Hoch, G. & Solter, L. F. 2006. *Vairimorpha disparis* n. comb. (Microsporidia: Burenellidae): A redescription of the *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) microsporidium, *Thelohania disparis* Timofejeva 1956. *J. Eukaryot. Microbiol.*, 53:292–304.
Vossbrinck, C. R. & Debrunner-Vossbrinck, B. A. 2005. Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. *Folia Parasitol.*, 52:131–142.
Vossbrinck, C. R., Debrunner-Vossbrinck, B. A. & Weiss, L. M. 2014. Phylogeny of the Microsporidia. *In:* Weiss, L. M. & Becnel, J. J. (ed.), Microsporidia: Pathogens of Opportunity. John Wiley and Sons, Inc., Ames, IA. p. 195–220.
Wang, J. Y., Huang, K. W., Mao, X. C., Zhao, Y. & Lu, C. D. 2001. Small subunit ribosomal RNA genes of Microsporidia. *Acta Biochim. Biophys. Sin.*, 33:229–232.
Weiser, J. 1961. Die mikiosporidien als parasiten der insekten. *Monogr. Angew. Entomol.*, 17:149 pp. (p. 60).
Xing, D., Li, L., Liao, S., Luo, G., Li, Q., Xiao, Y., Dai, F. & Yang, Q. 2014. Identification of a microsporidium isolated from *Megacopta cribraria* (Hemiptera: Plataspidae) and characterization of its pathogenicity in silkworms. *Antonie Van Leeuwenhoek*, 106:1061–1069.
Xu, X., Shen, Z., Zhu, F., Tao, H., Tang, X. & Xu, L. 2011. Phylogenetic characterization of a microsporidium (*Endoreticulatus* sp. Zhenjiang) isolated from the silkworm, *Bombyx mori*. *Parasitol. Res.*, 110:815–819.
Yu, Q., de Groot, P., Leal, I., Davis, C., Ye, W. & Foord, B. 2009. Characterization of *Deladenus siricidicola* (Tylenchida: Neotylenchidae) associated with *Sirex noctilio* (Hymenoptera, Siricidae) in Canada. *Int. J. Nematol.*, 19:23–32.
Zondag, R. 1969. A nematode infection of *Sirex noctilio* (F.) in New Zealand. *N. Z. J. Sci.*, 12:732–747.
Zondag, R. 1975. A non-sterilizing strain of *Deladenus siricidicola*. *Forest. Inst. Res. Rpt.*, 1974:51–52, Rotorua, NZ.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Alignment of named microsporidian small subunit sequences of close relatives to *Nosema bombycis* Naegeli and *Vairimorpha necatrix* Pilley.