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Morphological and molecular characterization of a microsporidian parasite, *Takaokaspora nipponicus* n. gen., n. sp. from the invasive rock pool mosquito, *Ochlerotatus japonicus japonicus*

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ABSTRACT

A new genus and species of Microsporidia, *Takaokaspora nipponicus* n. gen., n. sp. is described from *Ochlerotatus japonicus japonicus* (Theobald) and *Ochlerotatus hatorii* (Yamada) based on light microscope and ultrastructural morphology, developmental features, transmission cycles and comparative sequence analyses of the small subunit ribosomal DNA (SSU rDNA). The microsporidium is both vertically and horizontally transmitted, exhibits dimorphic development alternating between diplokaryotic and monokaryotic stages and produces two morphologically distinct spores, one in larvae and another in adult females. Horizontal transmission of infection to larval mosquitoes occurs via direct oral ingestion of uninucleate spores that are produced in vertically-infected larval hosts. Development in horizontally-infected hosts is diplokaryotic following karyokinesis of uninucleate schizonts and binary fission to produce small (4.3 μm × 2.0 μm) membrane free, ovoid, binucleate spores that are confined to adult female reproductive tissues (ovariole sheath and oviducts). Vertical transmission of the microsporidium from adult females to larval progeny takes place via surface contamination of the egg (transovum). Microsporidian development in vertically-infected larvae is haplophasic with unpaired nuclei throughout, producing rosette-shaped sporogonial plasmodia contained within a thin non-persistent sporophorous vesicle and culminating in the formation of membrane free, uninucleate, conical spores (7.0 μm × 2.8 μm). Development is confined to host fat body tissue which appears as swollen white masses in the thorax and selected segments of the abdomen causing larvae to appear abnormally distorted and results in death during the third and fourth instar stages. The SSU rDNA sequences obtained from the two morphologically identical microsporidia isolated from *Oc. j. japonicus* and *Oc. hatorii* were nearly identical and unique when compared with GenBank entries of all other mosquito-parasitic species. Phylogenetic trees constructed by Maximum Parsimony, Maximum Likelihood and bootstrap analyses using the Neighbor Joining search parameter yielded similar typologies. In each case, the novel microsporidium was the sister group to the clade containing *Parathelohania* species from *Anopheles* mosquitoes and the monotypic *Novothelohania ovalae* from *Ochlerotatus caspius* showing approximately 10–13% sequence divergence to those two genera providing strong support for establishment as a separate genus.

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1. Introduction

Ochlerotatus japonicus japonicus (Theobald) is an invasive rock hole and container-breeding mosquito native to East Asia that was first detected in the northeastern United States in 1998 (Peyton et al., 1999; Andreadis et al., 2001). It has rapidly spread throughout much of eastern North America where it is now firmly established, and appears to be a dominant invader that may be competitively displacing native species in natural rock hole and used tire habitats (Bevins, 2007; Andreadis and Wolfe, 2010). More recently, the species has invaded Canada (Thielman and Hunter, 2006) and central Europe (Schaffner et al., 2003; Schaffner et al., 2009; Versteirt et al., 2009; Medlock et al., 2012). It is presumed to have entered both the United States and Europe through the international trade in used automobile tires (Peyton et al. 1999; Schaffner et al., 2003; Versteirt et al., 2009; Medlock et al., 2012). The establishment of the species in these regions of the world is of considerable public health significance. It is an aggressive human biter (Molaei et al., 2009), is a competent vector of several important arboviruses including eastern equine encephalitis (Sardelis et al., 2002a) Japanese encephalitis (Takashima and Rosen,

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1989), LaCrosse virus (Sardelis et al., 2002b), and St. Louis encephalitis (Sardelis et al., 2003), and has been incriminated in transmission of West Nile virus in North America (Andreadis et al., 2001; Turell et al., 2001).

When an exotic species is introduced into a new region in the absence of any natural enemies that attack it in its native range, the likelihood of successful establishment and expansion of the invading species can be appreciably enhanced provided that species is able to cope with existing environmental conditions, effectively compete with resident species that occupy the same or similar ecological niche, and is not beset by native parasites or predators (Torchin et al., 2003; Juliano and Lounibos, 2005; Armistead et al., 2012). This phenomenon has been described as an "enemy release hypothesis" wherein invading organisms lose their co-evolved parasites and other natural enemies during the process of invasion leading to higher demographic success which in turn may lead to a competitive advantage for the invader over native species (Torchin et al., 2003; Prenter et al., 2004; Dunn, 2009). Accordingly, surveys of North American populations of *Oc. j. japonicus* have yet to uncover any significant parasites or predators. Reeves and Korecki (2004) noted the presence of a trichomycete fungus, *Smittium* sp. in the hindguts of larvae collected from rock pools in South Carolina. However, while there is some evidence that these fungi may cause blockage of the gut leading to death (Sweeney, 1981), most species of *Smittium* are generally considered to have a commensal relationship with their larval dipteran hosts and have little or no detrimental impact (Roberts and Panter, 1985).

In September of 1980, an unknown *Stempellia* – like microsporidian parasite producing "cone-shaped" spores was discovered infecting several early instar *Oc. j. japonicus* larvae collected from rock pools along the Okudake River, at the foot of Mt. Sobo in the Oita Prefecture, Kyushu Region of Japan (Takaoka, 1982). Infected larvae were recognized by the characteristic "whitish-appearance" of infected portions of fatbody tissue in the thorax and abdomen. Collections made in July of the following year revealed field infection rates ranging from 0.4% to 3.6%. Subsequent surveys made in 1984 and 1985 uncovered comparable infections with a similar microsporidium in cohabitating early instar *Ochlerotatus hatorii* (Yamada) larvae with infection rates ranging from 0.4% to 7.6% in *Oc. j. japonicus* and 0.3% to 4.2% in *Oc. hatorii* (Takaoka et al., 1986). However, detailed description and taxonomic status of the microsporidium were never determined.

In 2010, the original collection site in Japan was revisited in an attempt to recover this microsporidium and evaluate its potential as a biological control agent for possible introduction into North American populations of *Oc. j. japonicus*. The microsporidium was again found infecting larval populations of *Oc. j. japonicus* and *Oc. hatorii* and successfully isolated for analyses. In this investigation we now provide a complete morphological characterization of the life cycle this microsporidium by light and electron microscopy, demonstrate pathways of horizontal and vertical transmission in *Oc. j. japonicus*, and examine SSU rDNA sequence data to determine its unique placement among other mosquito-parasitic microsporidia. Based on morphological, ultrastructural and molecular evidence, we propose the creation of a new genus and species, *Takoakaspora nipponicus.*

2. Materials and methods

2.1. Source and collection of specimens

Second and third stage larval specimens of *Oc. j. japonicus* and *Oc. hatorii* infected with microsporidia were collected from riverside rock pools located along the upper portion of the Okudake River, at the foot of Mt. Sobo in the Oita Prefecture, Kyushu Region of Japan (32°49′43.91″N, 131°23′37.66″E; 560 m elev.) (Fig. 1). Collection dates for each species were as follows: *Oc. j. japonicus* – 7/29/10, 6/9/11, 9/7/11, 10/12/11, 8/30/12, 9/27/12; *Oc. hatorii* – 5/27/10, 6/23/10, 7/29/10, 8/31/10, 10/19/10, 6/9/11, 10/25/12, 11/15/12. Live specimens were rinsed in deionized water, individually isolated in vials, and immediately sent to laboratories at the Connecticut Agricultural Experiment Station for analysis.

2.2. Light microscope studies

General characterization of microsporidian development in both host mosquitoes was made from microscopic examination (1000×) of Giemsa-stained smears of infected tissues obtained from live and moribund larvae collected from the original field sites, and from larvae and adult *Oc .j. japonicus* infected in the laboratory transmission studies (see Section 2.5). Smears were air dried, fixed in 100% methanol (5 min), and stained with a 15% (v/v) modified Giemsa stain solution (pH 7.4) (20 min) (Sigma–Aldrich, St. Louis, MO). Tissue specificity was determined from histological examination of paraffin-embedded whole larval, adult male and female stages of *Oc. j. japonicus* procured from the laboratory transmission studies. These were stained with iron hematoxylin and eosin Y. Measurements of mature spores were calculated from examination of whole wet-mount preparations of live spores ($n = 50$) with differential interference optics in a Zeiss Axioplan 2 Digital imaging system (1000×).

2.3. Ultrastructural studies

The comparative ultrastructure of microsporidian development was performed in both host mosquitoes. Infected tissues from field-collected *Oc. j. japonicus* and *Oc. hatorii* larvae were fixed in a 2.5% (v/v) glutaraldehyde solution containing 0.1% (w/v) $CaCl_2$ and 1% (w/v) sucrose buffered in 100 mM Na cacodylate (pH 7.3) overnight at room temperature and postfixed in 1% (w/v) OsO_4 in the same buffer and temperature. Fixed specimens were dehydrated through a graded ethanol and acetone series and embedded in a LX-112/Araldite (Ladd Research Industries, Williston, VT) mixture. Thin sections (60–100 nm) were stained with 5% (w/v) uranyl acetate in 50% (v/v) methanol followed by Reynold's lead citrate, and examined in a Zeiss EM 10C electron microscope at an accelerating voltage of 80 kV.

2.4. Nucleic acid extraction, PCR amplification and sequencing of SSU rDNA

Independent nucleotide sequences were obtained from mature spores isolated from patently infected larvae of both *Oc. j. japonicus* and *Oc. hatorii*. Larval tissues were homogenized in 500 µl of sterile water, filtered through a 41 µm nylon mesh into a clean 1.5 ml microcentrifuge tube, and centrifuged at 14,000*g* for 2 min. The supernatant was removed, and 150 µl of STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to the spore pellet (Fluka, Buchs, Switzerland), resuspended and placed in a 0.5 ml microcentrifuge tube. A 10 µl aliquot of each sample was removed and examined by phase contrast microscopy (100×) for the presence of spores. One hundred fifty mg of glass beads (212–300 µm diameter) (Sigma, St. Louis, MO) were then added and the tube was shaken in a Mini-Beadbeater (Biospec Products Bartlesville, OK) for 50 s to fracture the spores. The samples were spun briefly and 10 µl was removed to verify spore disruption. The samples were incubated at 95 °C for 5 min, and then centrifuged at 14,000*g* for 5 min. The supernatant was removed to a clean 1.5 ml microcentrifuge tube and was frozen at −20 °C until use in PCR.

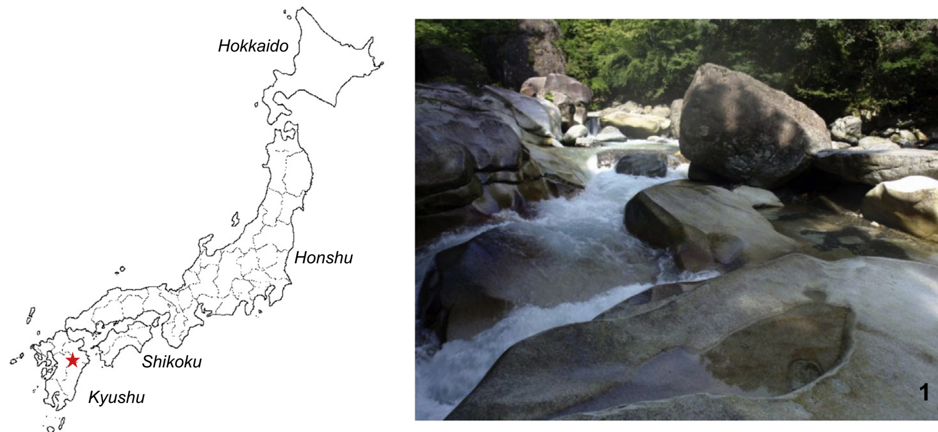


Fig. 1. Map of Japan showing collection site in the southernmost Kyushu region and rock pools along the Okudake River where the microsporidian infected *Oc. j. japonicus* and *Oc. hatorii* were found.

One to 5 µl of the STE-ruptured spore solution was used in a standard PCR reaction (94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 45 °C for 30 s, and 72 °C for 1 min 30 s) using primers 18f and 1492r (see below). The PCR product was then purified on a Qiaquick PCR purification kit (Qiagen Company, CA) and prepared for automated sequencing at the Keck Biotechnology Resource Laboratory at Yale University with the following microsporidian primers: 18f, CACCAGGTTGATTCTGCC; SS350f, CCAAGGAYGGCAGC AGGCGCGAAA; SS350r, TTTCGCGCCTGCTGCCRTCCTTG; SS530f, GTGCCAGCMGCCGCGG; SS530r, CCGCGGKGCTGGCAC; 1047r, AACGGCCATGCACCAC; 1061f, GGTGGTGCATGGCCG; 1492r, GGTTACCTTGTTACGACTT.

2.5. Phylogenetic analyses

SSU rDNA sequences of 49 microsporidian species (47 from mosquito hosts) obtained from our previous studies (Andreadis et al., 2012) and the NCBI Genbank database were selected for phylogenetic analysis (Table 1). This analysis included 4 outgroups representing the other microsporidial clades (Vossbrinck and DeBrunner-Vossbrinck, 2005): *Paranosema locustae* (clade 2), *Anncaliia (Brachiola) algerae* (clade 3), *Vairimorpha necatrix* (clade 4) and *Vavraia culicis* (clade 5). Sequences were aligned using the ClustalX program (Thompson et al., 1997) and then manually edited. Aligned sequences were analyzed by Maximum Parsimony, Maximum Likelihood and Neighbor Joining analyses using PAUP version 3.1b (Swofford, 1998). Bootstrap analysis was accomplished using 1000 Neighbor-joining replicates. Maximum Parsimony analysis was done using the heuristic search method. All characters were unordered and had equal weight, no topological constraints were enforced and 838 characters were parsimony informative. Maximum Likelihood analysis was accomplished using the heuristic search method.

2.6. Transmission studies

A series of horizontal transmission studies designed to qualitatively assess the oral infectivity of spores of the microsporidium to *Oc. j. japonicus* were conducted with a free mating laboratory colony originally established in 2000 from field collected larvae found in central New Jersey (Williges et al., 2008). Separate feeding trials were performed with spores acquired from field collected, naturally infected (1) *Oc. j. japonicus*, (2) *Oc. hatorii* and (3) laboratory infected *Oc. j. japonicus*. Each bioassay was performed at 25 °C under a 16:8 (LD) photoperiod in 100 × 80 mm culture dishes containing 100 ml of deionized water. Fifty second instar *Oc. j. japonicus* larvae were placed in each culture dish along with one heavily infected larval cadaver containing mature microsporidian spores (approximately 1×10^4 spores/larva). A small amount of an aqueous suspension of Brewer's yeast and liver powder (2:3 mixture) was provided for food. Developing larvae were visually screened for patent infections in black photographic pans and reared to adulthood. Dead and moribund individuals were examined for infection as whole wet-mount (phase contrast microscopy) or Giemsa-stained preparations (1000×) as described in Section 2.1.

Surviving adults were transferred to 30.3-cm^3 screened cages and held at 24 °C, 75% RH, 16:8 L:D photoperiod. They were maintained on a 10% sucrose solution and checked daily for mortality following which they were similarly assessed for infection. Females were allowed to blood feed on restrained guinea pigs and following oviposition or death were either examined directly to verify infection (Giemsa-stained smear) or processed for histology to determine tissue specificity. Adult males were correspondingly examined.

The potential for vertical transmission of the microsporidium was further determined by examining progeny reared from infected adult female *Oc. j. japonicus* that had been previously exposed as larvae to the microsporidium in the feeding trials. Eggs collected from these females were hatched approximately 4 wks after oviposition. Larvae were collectively reared in 180 by 290 mm white enamel pans at 25 °C under a 16:8 (LD) photoperiod as described above and visually examined for patent infections in black photographic pans. A cohort of larvae not showing visible signs of infection were reared to adulthood following which whole adults of both sexes were smeared on microscope slides, stained with Geisma and examined microscopically for infection.

3. Results

3.1. Vertically acquired larval infections

Larval mosquitoes with patent infections (field-collected and laboratory-induced) that were subsequently shown to be vertically acquired (see Section 3.3), possessed swollen white masses in the thorax and selected segments of the abdomen that caused larvae to appear abnormally distorted (Fig. 2). Infections were restricted to fatbody tissue and were not detected in any other internal structures that were examined in histological section including the alimentary canal, Malpighian tubules, musculature, oenocytes, nerve tissue, trachea, or developing testes or ovaries (Figs. 3 and 4). Mortality occurred prior to pupation during the third and fourth larval instar stages.

Table 1
Species list of microsporidian SSU rDNA sequences included in the phylogenetic analysis including host species and GenBank accession number.

Microsporidium species	Host species	Accession number
Amblyospora bakcharia	*Ochlerotatus excrucians*	JF826402
Amblyospora baritia	*Ochlerotatus excrucians*	JF826403
Amblyospora bogashova	*Ochlerotatus excrucians*	JF826404
Amblyospora californica	*Culex tarsalis*	U68473
Amblyospora canadensis	*Ochlerotatus canadensis*	AY090056
Amblyospora chulymia	*Ochlerotatus caspius*	JF826405
Amblyospora cinerei	*Aedes cinereus*	AY090057
Amblyospora connecticus	*Ochlerotatus cantator*	AF025685
Amblyospora criniferis	*Ochlerotatus crinifer*	AY090061
Amblyospora excrucii	*Ochlerotatus excrucians*	AY090043
Amblyospora ferocis	*Psorophora ferox*	AY090062
Amblyospora flavescens	*Ochlerotatus dianaetus*	JF826406
Amblyospora hristinia	*Ochlerotatus communis*	JF826407
Amblyospora indicola	*Culex sitiens*	AY090051
Amblyospora jurginia	*Ochlerotatus excrucians*	JF826408
Amblyospora kazankia	*Ochlerotatus dianaetus*	JF826409
Amblyospora khaliulini	*Ochlerotatus communis*	AY090045
Amblyospora kolarovi	*Ochlerotatus punctor*	JF826410
Amblyospora mavlukevia	*Aedes cinereus*	JF826411
Amblyospora mocrushinia	*Ochlerotatus punctor*	JF826412
Amblyospora modestium	*Culex modestus*	JF826413
Amblyospora opacita	*Culex territans*	AY090052
Amblyospora rugosa	*Ochlerotatus cataphylla*	JF826414
Amblyospora salairia	*Aedes cinereus*	JF826415
Amblyospora salinaria	*Culex salinarius*	U68474
Amblyospora severinia	*Ochlerotatus excrucians*	JF826417
Amblyospora shegaria	*Aedes cinereus*	JF826416
Amblyospora stictici	*Ochlerotatus sticticus*	AY090049
Amblyospora stimuli	*Ochlerotatus stimulans*	AF027685
Amblyospora timirasia	*Aedes cinereus*	JF826418
Amblyospora weiseri	*Ochlerotatus cantans*	AY090048
Andreanna caspii	*Ochlerotatus caspius*	EU664450
Anncaliia algerae	*Anopheles stephensi*	AF069063
Culicospora magna	*Culex restuans*	AY326269
Culicosporella lunata	*Culex pilosis*	AF027683
Edhazardia aedis	*Aedes aegypti*	AF027684
Hazardia milleri	*Culex quinquefasiatus*	AY090067
Hyalinocysta chapmani	*Culiseta melanura*	AF483837
Intrapredatorus barri	*Culex fuscanus*	AY013359
Novothelohania ovalae	*Ochlerotatus caspius*	JF826419
Parathelohania anophelis	*Anopheles quadrimaculatus*	AF027682
Parathelohania divulgata	*Anopheles messeae*	JF826420
Parathelohania obesa	*Anopheles crucians*	AY09006
Parathelohania tomski	*Anopheles messeae*	JF826421
Senoma globulifera	*Anopheles messeae*	DQ641245
Takaokaspora nipponicus	***Ochlerotatus japonicus japonicus***	**KF110990**
	Ochlerotatus hatorii	**KF110990**
Trichoctosporea pygopellita	*Ochlerotatus excrucians*	HM594267
Vavraia culicis	*Culex pipiens*	AJ252961
Paranosema locustae	Orthoptera	AY305325
Vairimorpha necatrix	Lepidoptera	Y0026

No differences in ultrastructural morphology of any vegetative stages or spores from either *Oc. j. japonicus* or *Oc. hatorii* were observed. The initial stages of development seen in Giemsa-stained smears were small (4–5 μm) oval, uninucleate schizonts (Fig. 5). In ultrastructure, they appeared fusiform (Fig. 13) delimited by a simple plasmalemma in direct contact with the host cell cytoplasm (Fig. 14). The cytoplasm of these schizonts was densely granular with numerous free ribosomes, stacks of endoplasmic reticulum and occasional vacuoles (Figs. 13–16). Spindle plaques and polar vesicles were frequently observed in association with the nucleus (Fig. 15), but neither Golgi apparatus nor mitosomes were detected. Schizonts underwent a series of synchronized nuclear divisions forming multinucleated cells with up to 16 unpaired individual nuclei (Figs. 5, 6 and 17, 18). We were unable to definitely determine if these multinucleated cells gave rise to daughter schizonts or not via cytokinesis. More frequently, we observed the development of a sporophorous vesicle surrounding these multinucleated cells that was accompanied by the concurrent formation of a lobbed rosette-shaped sporogonial plasmodia (up to 30 μm) with up to 24 unpaired nuclei (Figs. 7–9 and 19–21). The sporoph-

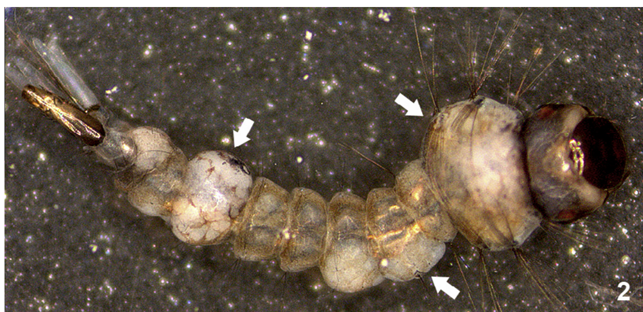
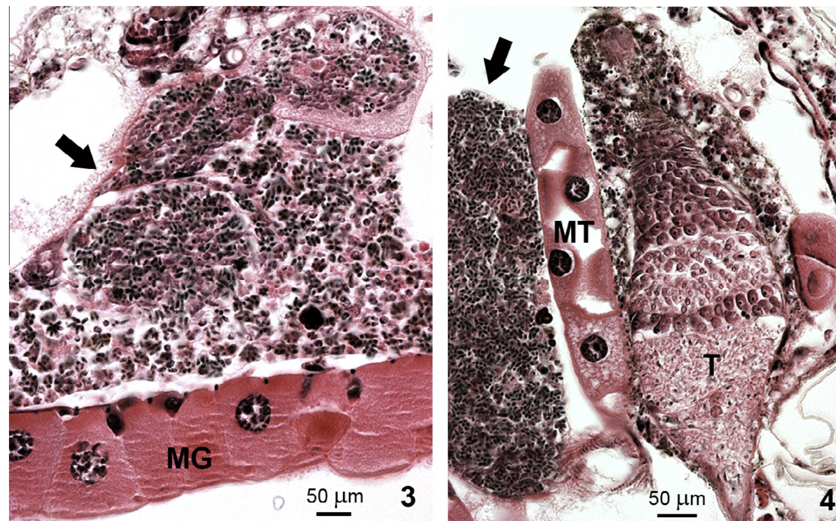
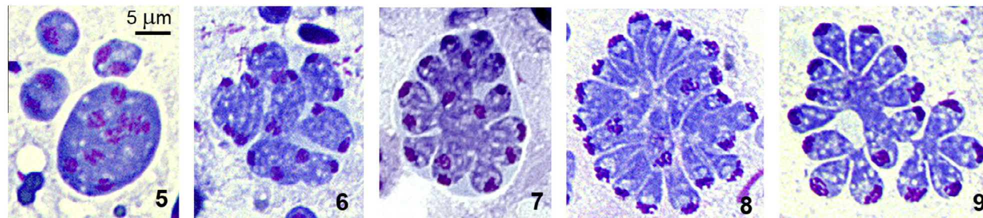


Fig. 2. Fourth instar *Oc. j. japonicus* larva displaying patent late stage, vertically-acquired, microsporidian infection in fat body tissue within the thorax and abdomen (arrows).



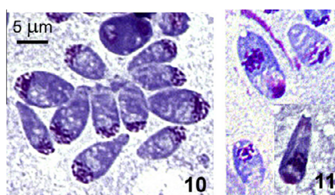
Figs. 3 and 4. Histological sections through the abdomen of a patently infected fourth instar *Oc. j. japonicus* larvae showing microsporidian infection in fat body tissue (arrows). MG, midgut epithelial cells; MT, Malpighian tubules; T, developing testes.



Figs. 5–9. Developmental stages of *Takaokaspora nipponicus* as observed in Giemsa-stained smears of patently infected *Oc. j. japonicus* larvae. (5) Uninucleate and multinucleate schizonts. (6) Multinucleated schizonts undergoing synchronous nuclear division. (7 and 8) Rosette-shaped sporogonial plasmodium within a sporophorous vesicle. (9) Sporogonial plasmodium undergoing cytoplasmic budding.

orous vesicle was seen as a thin non-persistent membrane enveloping the entire sporogonial plasmodium. Granular and crystalline inclusions contiguous with the cell plasmalemma were initially observed within the episporontal space (Figs. 19 and 20). These gradually disappeared with thickening of the plasmalemma and dissolution of the sporophorous vesicle (Figs. 9 and 21). Cytoplasmic division was by budding of the sporogonial plasmodium (Figs. 9 and 10) forming uninucleate sporoblasts (Figs. 11 and 22).

Mature spores were conical and averaged $7.0 \pm 0.4 \times 2.8 \pm 0.2$ μm (live) (mean ± SD) (Fig. 12). They were uninucleate and possessed a large posterior vacuole (Figs. 23 and 24). The polaroplast was voluminous and bipartite consisting of large irregularly spaced vesicular chambers in the anterior end and more tightly compressed lamellar elements in the posterior end (Figs. 24 and 25). The polar filament was isofilar with 3 coils and the anchoring disc of the polar sac was well developed (Figs. 25 and 26). The exospore was laminate, smooth and measured 31 nm, while the endospore measured 65 nm. Germinated spores and spores with extruding polar filaments (Fig. 27) were occasionally observed alongside other developing stages.



Figs. 10 and 11. Developmental stages of *Takaokaspora nipponicus* as observed in Giemsa-stained smears of patently infected *Oc. j. japonicus* larvae. (10) Group of non-membrane bound early stage sporoblasts immediately following cytoplasmic budding of the sporogonial plasmodium. (11) Uninucleate sporoblasts undergoing sporogenesis.

3.2. Horizontally acquired infections

Late instar larval *Oc. j. japonicus* that were exposed as second instars to spores procured from patently infected dead and dying larvae with patent infections , were found to harbor a few small

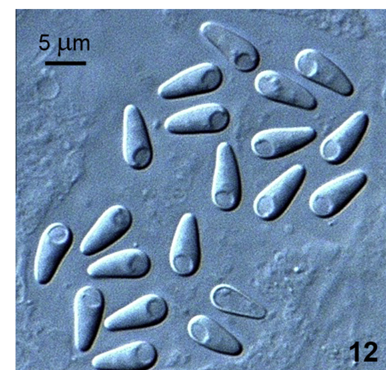
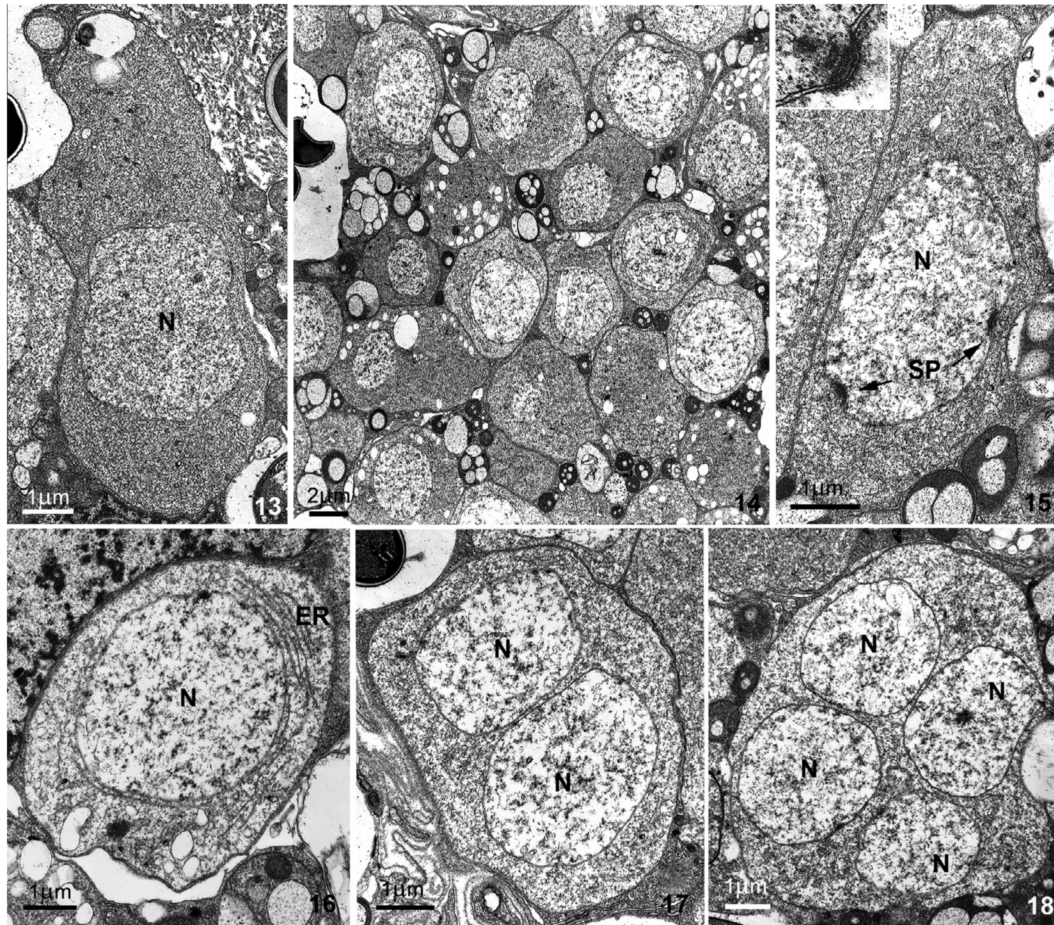


Fig. 12. Fresh live spores of *Takaokaspora nipponicus* from a patently infected *Oc. j. japonicus* larva (differential interference contrast).



Figs. 13–18. Transmission electron micrographs of early schizogonic stages of *Takaokaspora nipponicus* development in fat body tissues of vertically-infected infected *Oc. j. japonicus* larvae. (13) Early fusiform shaped uninucleate schizont. (14) Tightly clustered group of uninucleate schizonts interspersed among electron dense lipid inclusions. (15) Uninucleate schizont displaying spindle plaques (SP) associated with nuclear division (inset 42,000×). (16) Uninucleate schizont with well-developed endoplasmic reticulum (ER). (17) Binucleate schizont. (18) Multinucleated schizont. N, nucleus.

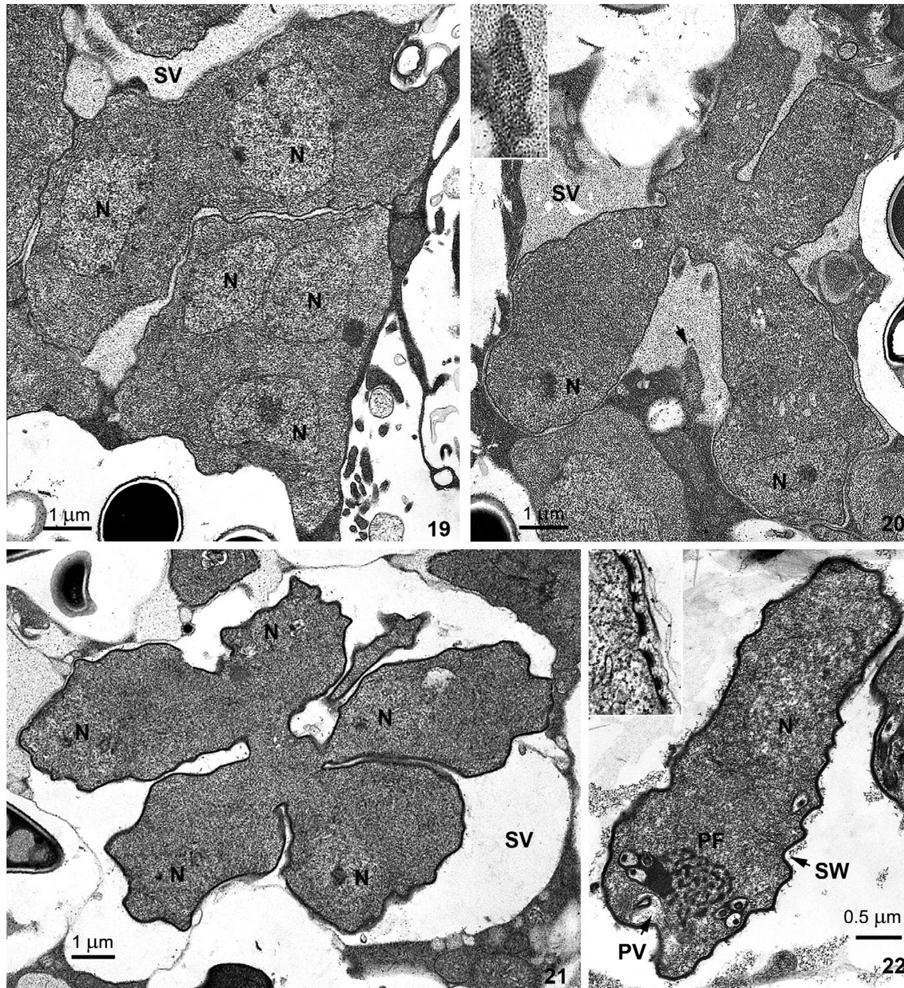
(5 μm) uninucleate schizonts that were mostly fusiform with a nucleus at one pole (Figs. 28 and 29). No evidence of plasmogamy was detected, but instead schizonts appeared to undergo nuclear division (karyokinesis) (Fig. 30) to form diplokaryotic meronts (Fig. 31). These stages were identified most frequently in pupae and adults and were very few in number. Diplokaryotic meronts in the process of undergoing synchronous binary fission (merogony) that resulted in the creation of two diplokaryotic daughter cells (sporonts) were also noted (Figs. 32 and 34). While a few vegetative stages were seen in smears of adult males, sporulation and sporogenesis of diplokaryotic sporonts were observed in adult females only. This resulted in the formation of a comparatively large number of small ovoid spores (4.3 ± 0.5 μm × 2.0 ± 0.2 μm) (fixed) (mean ± SD) (Fig. 35) that were apparent in Giemsa-stained smears of female abdomens. Although we were unable to perform ultrastructural examination of this phase of development, observations of sporoblasts and spores under the light microscope (1000×) provided clear evidence that these small ovoid spores were binucleate.

3.3. Vertical transmission studies

Vertical transmission of the microsporidium from infected adult female *Oc. j. japonicus* that had been previously exposed as larvae to the microsporidium in the feeding trials described in Section 3.2, was demonstrated on two separate occasions. In the first trial, patent fatbody infections were detected in 32 of 104 (30.8%) F1 progeny that were hatched and reared from eggs collected from a cohort of adult females where 78.6% ($n = 14$) of these females were found to be infected. Histological examination of these parental females revealed heavy infections with spores within the lateral oviduct (Fig. 36) and ovariole sheath surrounding the follicular epithelium and chorion of the mature oocytes (Fig. 37). No infections were detected within the nurse cells or yolk, nor were they observed in any other host tissues. In the second trial, patent fatbody infections were detected in 11 of 51 (21.6%) F1 progeny from a cohort of adult females where 21.6% ($n = 30$) of these females were similarly found to be infected. All infected F1 progeny were found to harbor patent fatbody infections resulting in the production of uninucleate conical spores as described in Section 3.1.

3.4. Molecular phylogenetic analysis

The SSU rDNA sequences (approximately 1260 bp) obtained from the two morphologically identical microsporidia isolated from *Oc. j. japonicus* (GenBank accession number KF110990) and *Oc. hatorii* (GenBank accession number KF110991) were nearly identical and unique when compared with GenBank entries of all other mosquito-parasitic species. Phylogenetic trees constructed by Maximum Parsimony (not shown), Maximum Likelihood (not shown) and bootstrap analyses using the Neighbor Joining search parameter (1000 replicates) (Fig. 38) yielded similar typologies. In each case, the novel microsporidium was



Figs. 19–22. Transmission electron micrographs of sporogonic stages of *Takaokaspora nipponicus* development in fat body tissues of vertically-infected *Oc. j. japonicus* larvae. (19) Multinucleated transitional sporonts. (20) Early stage sporogonial plasmodium displaying granular and crystalline inclusions (arrow and inset) within the episporontal space of the sporophorous vesicle (SV) (inset 18,000×). (21) Late stage rosette-shaped sporogonial plasmodium contained within a thin sporophorous vesicle devoid of any episporontal inclusion prior to sporogenesis. (22) Sporoblasts. Inset showing early deposition of the spore wall (44,000×). N, nucleus; PV, posterior vacuole; SW, spore wall.

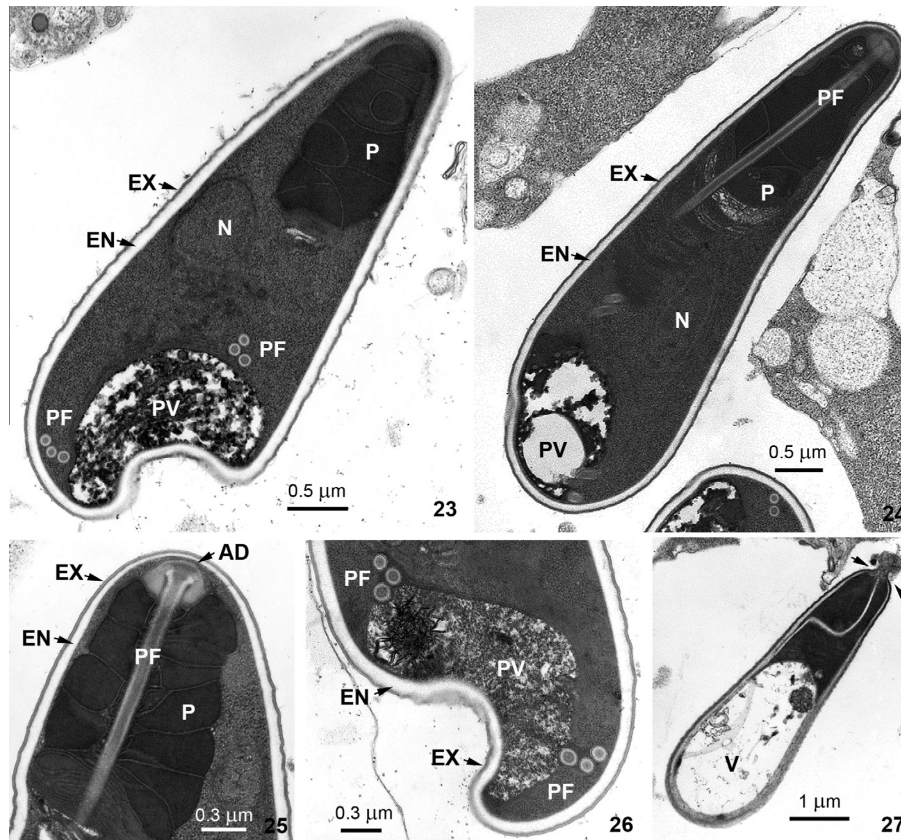
the sister group to the clade containing *Parathelohania* species from *Anopheles* mosquitoes and the monotypic *Novothelohania ovalae* from *Ochlerotatus caspius* (94–100% bootstrap support) showing approximately 87–90% sequence similarity to those two genera (Table 2). The newly proposed microsporidium was most closely related to *Parathelohania obesa* (89.9% sequence identity) but differed by approximately 127 nucleotides, a comparatively large number. Differences with other genera ranged from 17% to 32% (Table 2).

4. Discussion

4.1. Justification for creation of new genus

The unique morphological characters observed in spores and correspondingly novel SSU rDNA sequences obtained from this microsporidium isolated from *Oc. j. japonicus* and *Oc. hatorii* support its designation as a new genus and species within clade I (fresh water origin) of Vossbrinck and Debrunner-Vossbrinck (2005). We accordingly propose the following scientific name, *Takaokaspora nipponicus* n. gen., n. sp. in recognition of the original discovery by Hiroyuki Takaoka. This brings to 25 the number of microsporidian genera described from mosquitoes, 14 of which are monotypic, and only 11 of which molecular data are available for phylogenetic analysis (Andreadis, 2007; Simakova et al., 2008; Andreadis et al., 2012).

We found no contradictions between morphological and ultrastructural parameters and genetic relatedness. *T. nipponicus* segregated as a sister group to *N. ovalea* and the *Parathelohania* clade from *Anopheles* mosquitoes but exhibited a sequence divergence of more than 10% (~127 nucleotides) with its nearest relative, *P. obesa* (Table 2). Moreover, spores of this microsporidium exhibited unique ultrastructural morphology not seen in any other mosquito-parasitic genera, and possessed none of the diagnostic morphological features associated with *Parathelohania* meiospores, most notably, the posterior "bottleneck" extension of the spore wall and abruptly constricted anisofilar polar filament (Hazard and Anthony, 1974), nor were they similar in size, shape or internal structure to the thick walled oval uninucleate "octospores" with a large umbrella-shaped anchoring disc, tightly lamellate polaroplast and tapered polar filament found in *N. ovalea* (Andreadis et al., 2012). While we do not support a hard fast rule defining taxonomic differences with sequence similarity, it appears that within this clade of mosquito-parasitic species, SSU rDNA sequence similarity below the 90–92% level correlates with corresponding morphological and ultrastructural differences that warrant different generic designations (Table 2).

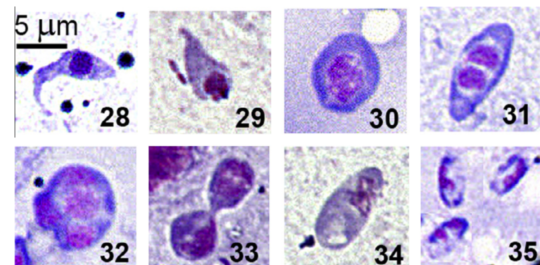


Figs. 23–27. Transmission electron micrographs of mature spores of *Takaokaspora nipponicus* from fat body tissues of vertically-infected infected *Oc. j. japonicus* larvae. (27) Germinating spore with extruding polar filament (arrows). AD, anchoring disc; EN, Endospore; EX, exospore; N, nucleus; P, polaroplast; PF, polar filament; PV posterior vacuole; V, vacuole.

4.2. Interpretation of the life cycle

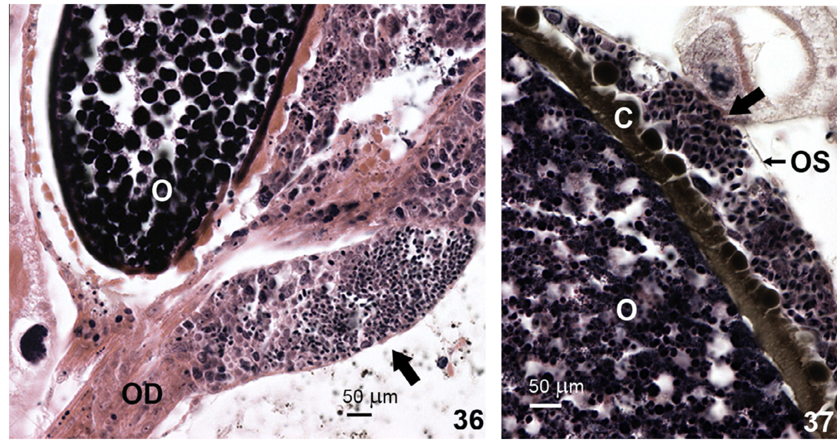
Our interpretation of the life cycle of *T. nipponicus* in *Oc. j. japonicus* and *Oc. hatorii* is depicted in Fig. 39. The microsporidium is both vertically and horizontally transmitted, exhibits dimorphic development alternating between diplokaryotic and monokaryotic stages, produces two morphologically distinct spores, one in larvae and another in adult females, and does not require an intermediate host. Horizontal transmission of infection occurs in larval mosquitoes via direct oral ingestion of uninucleate spores that are produced in late stage larvae and presumably released into the aquatic environment with the death and decay of these individuals. Spore germination likely takes place in the lumen of the gut and the first developmental stages found in exposed larvae are small uninucleate schizonts. We were unable to determine the initial site of infection but found no evidence for the formation of an autoinfective spore that functions in dispersal of infection as seen in some other microsporidia such as *Edhazardia aedis* (Johnson et al., 1997). Rather, we find very limited multiplication and no proliferation of infection in immature hosts.

As larvae develop, uninucleate schizonts undergo karyokinesis forming diplokaryotic meronts in a process previously thought to be unique but similarly observed in *Hyalinocysta chapmani* (Andreadis and Vossbrinck, 2002). The creation of the diplokaryotic condition in these two species is markedly different from that documented in most other microsporidia (e.g. *Culicospora magna*, *E. aedis*, *Hazardia milleri*, *Amblyospora* spp.), where diplokaryotic meronts are alternatively formed by cytoplasmic pairing (plasmogamy) and nuclear association of uninucleated gametes (Andreadis, 1985a, 1988; Hazard et al., 1985; Becnel et al., 1987, 1989; Becnel, 1992; Becnel and Andreadis, 1998) in what is interpreted as a sexual phase of development. Diplokaryotic meronts of this new species then exhibit a proliferative phase of development during which they repeatedly divide by binary fission to produce identical daughter cells. This asexual phase of multiplication occurs in both host sexes but extensive build-up of infection is restricted to adult females and does not ensue until after adult emergence. Sporogenesis follows resulting in the formation of a comparatively large number of small binucleate spores that are exclusive to females and are confined to reproductive tissues (i.e. ovariole sheath, oviducts).



Figs. 28–35. Developmental stages of *Takaokaspora nipponicus* as observed in Giemsa-stained smears of orally infected *Oc. j. japonicus*. (28 and 29) Early stage uninucleate schizonts found in larvae. (30) Transitional meront undergoing karyokinesis. (31) Diplokaryotic meront found in pupae and adults. (32) Multinucleated meront. (33) Multinucleated meront undergoing binary fission. (34) Binucleate sporoblast found in adult female. (35) Mature binucleate spores from adult females.

Vertical transmission of the microsporidium from adult females to larval progeny appears to take place via surface contamination



Figs. 36 and 37. Histological sections through the abdomen and reproductive tissues of an adult female *Oc. j. japonicus* showing binucleate spore infection (arrows) with *Takaokaspora nipponicus* in oviducts (OD) and within the ovarian sheath (OS) adjacent to the chorion (C) of the mature oocyte (O).

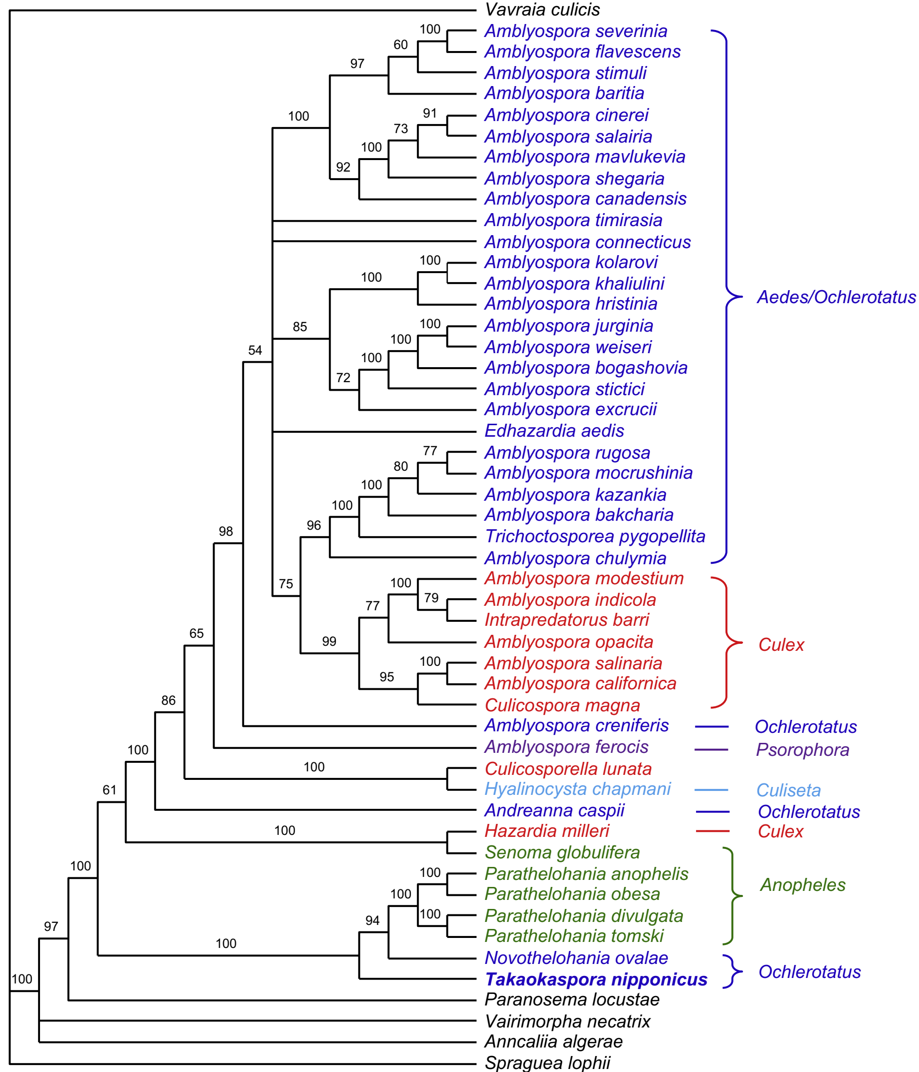


Fig. 38. Phylogenetic tree inferred from partial SSUrDNA gene sequences showing relationship of *Takaokaspora nipponicus* with representative species from 14 other mosquito-parasitic genera from which molecular data are available. Bootstrap analysis based on 1000 replicates using Neighbor Joining analysis. Numbers above branches indicate bootstrap support.

of the egg (transovum) as we could find no evidence of microsporidial development within the oocytes as seen in other mosquito-parasitic genera such as *Amblyospora*, *Culicospora*, *Edhazardia*, and *Parathelohania* where true transovarial transmission takes place (Hazard and Weiser, 1968; Andreadis and Hall, 1979; Andreadis, 1983, 2007; Becnel et al., 1987, 1989). However, efforts should

Table 2
Pairwise distance matrix showing the percent similarities between *Takaokaspora nipponicus* and representative species of 14 genera of mosquito-parasitic microsporidia based on SSU rDNA sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *Takaokaspora nipponicus*	–															
2. *Parathelohania obesa*	**89.9**	–														
3. *Novothelohania ovalae*	**87.4**	90.7	–													
4. *Hazardia milleri*	**82.9**	82.9	82.0	–												
5. *Amblyospora ferocis*	**80.9**	80.1	84.2	85.4	–											
6. *Andreanna caspii*	**80.8**	82.1	88.0	88.0	89.0	–										
7. *Senoma globulifera*	**80.7**	80.8	79.6	91.7	84.2	87.5	–									
8. *Amblyospora stictici*	**80.3**	80.8	80.2	83.7	91.3	87.4	81.1	–								
9. *Intrapredatorus barri*	**79.9**	80.1	80.0	83.9	91.0	87.8	83.9	93.1	–							
10. *Amblyospora indicola*	**79.3**	79.5	80.0	84.1	90.7	87.5	83.5	92.2	97.4	–						
11. *Trichoctosporea pygopellita*	**79.1**	79.5	80.5	84.4	90.7	88.4	82.1	93.7	95.2	94.4	–					
12. *Culicospora magna*	**79.0**	79.0	79.1	84.2	89.0	86.2	82.1	91.6	93.7	93.7	92.9	–				
13. *Edhazardia aedis*	**78.9**	79.1	78.9	83.6	90.4	87.4	82.1	94.1	94.1	94.5	94.6	92.8	–			
14. *Culicosporella lunata*	**77.3**	78.1	78.9	80.1	84.9	85.1	79.0	86.6	85.4	85.6	85.4	84.7	86.1	–		
15. *Hyalinocysta chapmani*	**77.2**	77.6	76.3	79.5	85.1	82.9	79.1	85.5	85.6	85.7	86.5	84.4	86.5	88.6	–	
16. *Vavraia culicis*	**71.1**	72.4	70.5	72.1	71.0	72.8	70.6	71.3	70.7	70.9	71.1	70.6	70.6	71.0	70.3	–
17. *Anncaliia algerae*	**67.8**	67.2	66.0	68.5	69.1	68.1	66.8	67.4	67.2	67.2	66.6	66.8	67.3	67.2	66.6	70.3

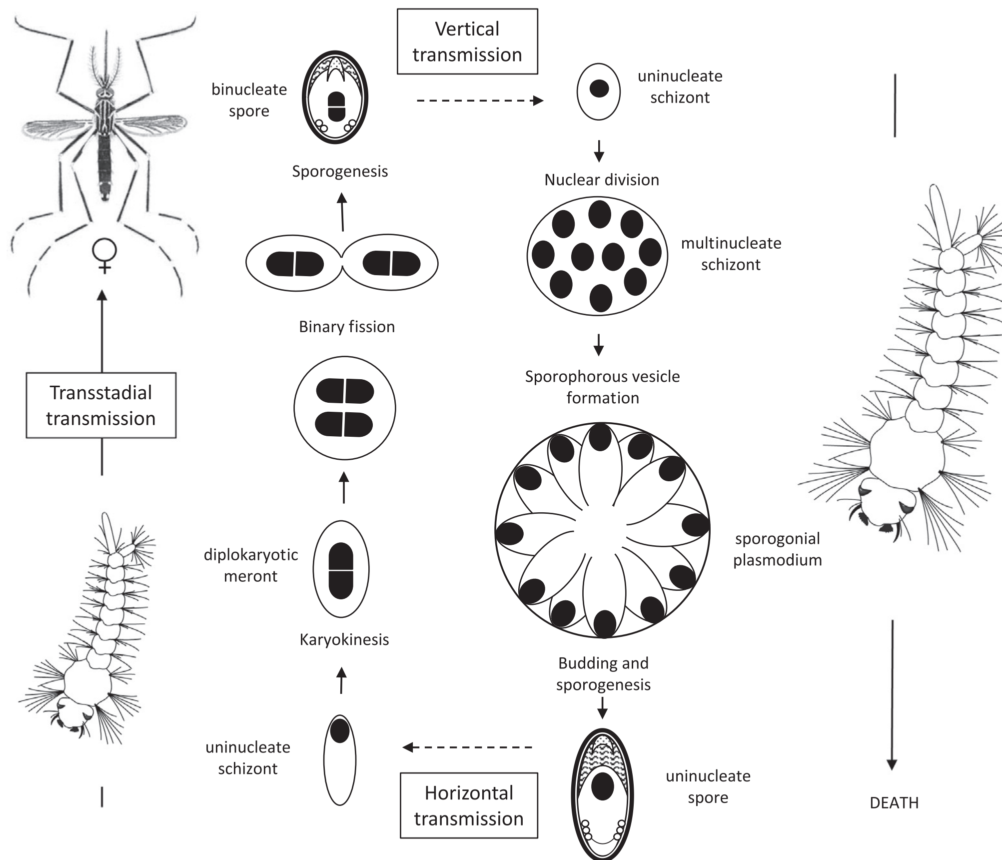


Fig. 39. Putative life cycle of *Takaokaspora nipponicus*, inferred from sequential light, ultrastructural, and histological observations of life stages and both horizontal and vertical transmission events in *Ochlerotatus j. japonicus*.

be made to surface sterilize mosquito eggs from infected females to fully verify this observation. Although we did not measure the impact of these infections on adult female survival or reproductive capacity, we did not observe any overt morbidity or premature mortality. Nonetheless, this also remains to be assessed as does the individual rate of transmission by infected females, as the latter appears to be comparatively inefficient.

Microsporidian development in vertically infected larvae is haplophasic with unpaired nuclei throughout, and is confined to host fat body tissue. Uninucleate schizonts, presumably derived from the germination of binucleate spores, are the first developmental stages observed, but it is unclear how the monokaryotic condition is attained. It most likely arises via nuclear dissociation of each member of the diplokaryon followed by cytoplasmic division as detailed in at least three other mosquito-parasitic species, *Amblyospora trinus* (Becnel and Sweeney, 1990), *C. magna* (Becnel et al., 1987) and *E. aedis* (Becnel et al., 1989). Alternatively, these uninucleate schizonts could be derived from nuclear fusion (karyogamy), but this process is typically followed by meiotic division and a prolonged sporulation sequence as seen in *Amblyospora*,

Hyalinocysta and *Parathelohania* (Hazard et al., 1979; Andreadis, 1983; Hazard and Brookbank, 1984; Andreadis and Vossbrinck, 2002).

Schizonts undergo repeated nuclear division forming multinucleated cells with up to 16 unpaired nuclei. These multinucleated schizonts do not appear to undergo any cytoplasmic fragmentation (plasmotomy) or multiple budding (schizogony) (Larsson, 1986), but rather it is here we observe the secretion of a thin sporophorous vesicle without ornamentation which we interpret as the initiation of sporogony. This is accompanied by further nuclear division and the formation of a lobbed rosette-shaped sporogonial plasmodium with varying numbers of unpaired nuclei up to 24. This is followed by disintegration of the sporophorous vesicle which uncharacteristically takes place prior to cytoplasmic cleavage (budding) of individual sporoblasts. Sporogenesis ensues in the typical manner with differentiation of the endospore and exospore, and concurrent assembly of the polar filament and anchoring disc, polaroplast, and posterior vacuole resulting in the formation of membrane-free uninucleate spores that are morphologically unique from those found in other mosquito-parasitic groups. Development of *T. nipponicus* in *Oc. j. japonicus* larvae differs greatly from the developmental sequences found in larval hosts other mosquito-parasitic genera such as *Amblyospora*, *Duboscqia*, *Hyalinocysta*, and *Parathelohania* which include meiotic division of diplokaryotic meronts and the formation of eight uninucleate spores contained within a persistent sporophorous vesicle. However, we note that*T. nipponicus* does exhibit several similarities (haplophasic development, formation of sporogonial plasmodia, and uninucleate spores) with development of the latter groups in their respective intermediate copepod host (Andreadis, 1985a,b; Avery and Undeen, 1990; Becnel, 1992; Sweeney et al., 1993; Micieli et al., 1998, 2000a, 2000b; Andreadis and Vossbrinck, 2002).

The routine detection of germinating and evacuated spores leads us to conclude that in addition to being orally infectious to larvae, these spores are involved in intrahost dissemination of infection within fat body cells. This again is in stark contrast to development of most other mosquito-parasitic microsporidia where cell to cell progression of infection proceeds within the fat bodies of larval hosts prior to spore formation and in the absence of internally germinating spores (Andreadis and Hall, 1979; Vavra and Lukes, 2013). Notable exceptions include: (1) *A. algerae* where morphologically similar first generation spores formed in the midgut of anopheline larvae, germinate soon after maturity to infect other tissues (Avery and Anthony, 1983), and (2) *E. aedis* which produces a morphologically distinct binucleate spore in gastric caecal cells of *Ae. aegypti* larvae that is responsible for dispersal of infection to other tissues where another morphologically and functionally distinct spore responsible for transovarial transmission is formed (Johnson et al., 1997). It is of interest to note that these and other "precociously germinating" spores have comparatively thin spore walls, large posterior vacuoles and short polar filaments (Vavra and Lukes, 2013) as similarly found in *T. nipponicus.*

5. Taxonomic summary

5.1. *Takaokaspora n. g*

5.1.1. *Definition*

Monotypic genus exhibiting dimorphic development alternating between diplokaryotic and monokaryotic stages and producing two morphologically distinct spores. Development in vertically-infected larval hosts is haplophasic with unpaired nuclei throughout, producing rosette-shaped sporogonial plasmodia contained within a thin non-persistent sporophorous vesicle and culminating in the formation of membrane free, uninucleate, conical spores. Development in horizontally-infected hosts is diplokaryotic following karyokinesis of uninucleate schizonts and binary fission to produce membrane free binucleate spores.

5.2. *Takaokaspora nipponicus Andreadis, Takaoka, Otsuka & Vossbrinck n. sp*

5.2.1. *Specific diagnosis*

With characters of the genus. Live mature spores from larval hosts conical, uninucleate, with large posterior vacuole, $7.0 \pm 0.4\ \mu m \times 2.8 \pm 0.2\ \mu m$ (mean ± SD) in size. Polaroplast voluminous and bipartite consisting of large irregularly spaced vesicular chambers in the anterior end and more tightly compressed lamellar elements in the posterior end. Polar filament isofilar with 3 irregularly arranged coils. Anchoring disc of the polar sac well developed. Exospore laminate, smooth and thin (31 nm) with thicker endospore (65 nm). Fixed mature spores in adult female hosts ovoid and binucleate measuring $4.3 \pm 0.5\ \mu m \times 2.0 \pm 0.2\ \mu m$ (mean ± SD).

Type host: *Ochlerotatus japonicus japonicus* (Theobald) (Diptera: Culicidae). *Additional host: Ochlerotatus hatorii* (Yamada) (Diptera, Culicidae)

Type locality: River-side rock pools located along the Okudake River at the foot of Mt. Sobo, Oita Prefecture, Kyushu Region of Japan (32°49′43.91″N, 131°23′37.66″E; 560 m elev.)

Site of infection: Fat body tissue of vertically-infected larval hosts. Reproductive tissues (ovariole sheath and oviducts) of horizontally-infected adult female hosts.

Transmission: Horizontal to larval hosts via oral ingestion of uninucleate spores from vertically-infected larval hosts. Vertical (transovum) from adult female to progeny via surface contamination of egg with binucleate spores.

Etymology: The genus is named in recognition of the original discovery of the microsporidium by Dr. Hiroyuki Takaoka, Professor Emeritus, Oita University.

Type material: Holotype and syntype slides consisting of: (1) Giemsa-stained smears and paraffin embedded histological sections of infected tissues from larval and adult *Oc. j. japonicus,* (2) embedded tissues from larval *Oc. j. japonicus* and *Oc. hatorii* used in the ultrastructural investigations and (3) frozen DNAs of the microsporidium from each host mosquito are in the collection of Theodore G. Andreadis, Center for Vector Biology, The Connecticut Agricultural Experiment Station, New Haven CT.

Gene sequences: The SSU rDNA sequences of the microsporidium, *T. nipponicus* have been deposited in the GenBank/EMBL database under Accession nos. KF110990 (*Oc. j. japonicus*) and KF110991 (*Oc. hatorii*).

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References

Andreadis, T.G., 1983. Life cycle and epizootiology of *Amblyospora* sp. (Microspora: Amblyosporidae) in the mosquito, *Aedes cantator*. J. Protozool. 30, 509–518.

Andreadis, T.G., 1985a. Life cycle, epizootiology and horizontal transmission of *Amblyospora* (Microspora: Amblyosporidae) in a univoltine mosquito, *Aedes stimulans*. J. Invertebr. Pathol. 46, 31–46.

Andreadis, T.G., 1985b. Experimental transmission of a microsporidian pathogen from mosquitoes to an alternate copepod host. Proc. Nat. Acad. Sci. 82, 5574–5577.
Andreadis, T.G., 1988. *Amblyospora connecticus* sp. nov. (Microsporida: Amblyosporidae): horizontal transmission studies in the mosquito, *Aedes cantator* and formal description. J. Invertebr. Pathol. 52, 90–101.
Andreadis, T.G., 2007. Microsporidian parasites of mosquitoes. In: Floore, T.G. (Ed.), Biorational Control of Mosquitoes, Bull. No. 7, Am. Mosq. Control Assoc. vol. 23, pp. 3–29.
Andreadis, T.G., Hall, D.W., 1979. Development, ultrastructure, and mode of transmission of *Amblyospora* sp. (Microspora: Thelohaniidae) in the mosquito. J. Protozool. 26, 444–452.
Andreadis, T.G., Vossbrinck, C.F., 2002. Life cycle, ultrastructure and molecular phylogeny of *Hyalinocysta chapmani* (Microsporidia: Thelohaniidae) a parasite of *Culiseta melanura* (Diptera: Culicidae) and *Orthocyclops modestus* (Copepoda: Cyclopidae). J. Eukaryot. Microbiol. 49, 350–364.
Andreadis, T.G., Wolfe, R.J., 2010. Evidence for reduction of native mosquitoes with increased expansion of invasive *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) in the northeastern United States. J. Med. Entomol. 47, 43–52.
Andreadis, T.G., Anderson, J.F., Munstermann, L.E., Wolfe, R.J., Florin, D.A., 2001. Discovery, distribution, and abundance of the newly introduced mosquito *Ochlerotatus japonicus* (Diptera: Culicidae) in Connecticut, USA. J. Med. Entomol. 38, 774–779.
Andreadis, T.G., Simakova, A.V., Vossbrinck, C.R., Shepard, J.J., Yurchenko, Y.A., 2012. Ultrastructural characterization and comparative phylogenetic analysis of new Microsporidia from Siberian mosquitoes: evidence for coevolution and host switching. J. Invertebr. Pathol. 109, 59–75.
Armistead, J.S., Arias, J.R., Nishimura, N., Lounibos, L.P., 2012. Community ecology of container mosquitoes (Diptera:Culicidae) in Virginia following invasion by *Aedes japonicus*. J. Med. Entomol. 49, 1318–1327.
Avery, S.W., Anthony, D.W., 1983. Ultrastructural study of early development of *Nosema algerae* in *Anopheles albimanus*. J. Invertebr. Pathol. 42, 87–95.
Avery, S.W., Undeen, A.H., 1990. Horizontal transmission of *Parathelohania anophelis* to the copepod, *Microcyclops varicans,* and the mosquito, *Anopheles quadrimaculatus*. J. Invertebr. Pathol. 56, 98–105.
Becnel, J.J., 1992. Horizontal transmission and subsequent development of *Amblyospora californica* (Microsporida: Amblyosporidae) in the intermediate and definitive hosts. Dis. Aquat. Org. 13, 17–28.
Becnel, J.J., Andreadis, T.G., 1998. *Amblyospora salinaria* n. sp. (Microsporidia: Amblyosporidae): parasite of *Culex salinarius* (Diptera: Culicidae), its life stages in an intermediate host and establishment as a new species. J. Invertebr. Pathol. 71, 258–262.
Becnel, J.J., Sweeney, A.W., 1990. *Amblyospora trinus* N. Sp. (Microsporidia: Amblyospridae) in the Australian mosquito *Culex halifaxi* (Diptera: Culicidae). J. Protozool. 37, 584–592.
Becnel, J.J., Hazard, E.I., Fukuda, T., Sprague, V., 1987. Life cycle of *Culicospora magna* (Kudo, 1920) (Microsporida: Culicosporidae) in *Culex restuans* Theobald with special reference to sexuality. J. Protozool. 34, 313–322.
Becnel, J.J., Sprague, V., Fukuda, T., Hazard, E.I., 1989. Development of *Edhazardia aedis* (Kudo, 1930) n. g., n. comb. (Microsporida: Amblyosporidae) in the mosquito *Aedes aegypti* (L.) (Diptera: Culicidae). J. Protozool. 36, 119–130.
Bevins, S.N., 2007. Establishment and abundance of a recently introduced mosquito species *Ochlerotatus japonicus* (Diptera: Culicidae) in the Southern Appalachians, USA. J. Med. Entomol. 44, 945–952.
Dunn, A.M., 2009. Parasites and biological invasions. Adv. Parasitol. 68, 161–184.
Hazard, E.I., Anthony, D.W., 1974. A redescription of the genus *Parathelohania* Codreanu 1966 (Microsporida: Protozoa) with a reexamination of previously described species of *Thelohania* Henneguy 1892 and descriptions of two new species of *Parathelohania* from anopheline mosquitoes. US. Dept. Agric. Technol. Bull. 1505, 1–26.
Hazard, E.I., Brookbank, J.W., 1984. Karyogamy and meiosis in an *Amblyospora* sp. (Microspora) in the mosquito, *Culex salinarius*. J. Invertebr. Pathol. 44, 3–11.
Hazard, E.I., Weiser, J., 1968. Spores of *Thelohania* in adult female *Anopheles*: development and transovarial transmission, and redescriptions of *T. legeri* Hesse and *T. obesa* Kudo. J. Protozool. 15, 817–823.
Hazard, E.I., Andreadis, T.G., Joslyn, D.J., Ellis, E.A., 1979. Meiosis and its implications in the life cycles of *Amblyospora* and *Parathelohania* (Microspora). J. Parasitol. 65, 117–122.
Hazard, E.I., Fukuda, T., Becnel, J.J., 1985. Gametogenesis and plasmogomy in certain species of Microspora. J. Invertebr. Pathol. 46, 63–69.
Johnson, M.A., Becnel, J.J., Undeen, A.H., 1997. A new sporulation sequence in *Edhazardia aedis* (Microsporidia: Culicosporidae), a parasite of the mosquito *Aedes aegypti* (Diptera: Culicidae). J. Invertebr. Pathol. 70, 69–75.
Juliano, S.A., Lounibos, L.P., 2005. Ecology of invasive mosquitoes: effects on resident species and on human health. Ecol. Lett. 8, 558–574.
Larsson, R., 1986. Ultrastructure, function and classification of microsporidia. Prog. Protistol. 1, 325–390.
Medlock, J.M., Hansford, K.M., Schaffner, F., Versteirt, V., Hendrickx, G., Zeller, H., Van Bortel, W., 2012. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector-Borne Zoonotic Dis. 12, 435–446.
Micieli, M.V., Garcia, J.J., Becnel, J.J., 1998. Horizontal transmission of *Amblyospora dolosi* (Microsporidia: Amblyosporidae) to the copepod *Metacyclops mendocinus* (Wierzejski, 1892). J. Invertebr. Pathol. 72, 330–335.
Micieli, M.V., Garcia, J.J., Becnel, J.J., 2000a. Life cycle and description of *Amblyospora camposi* n. sp. (Microsporidia: Amblyosporidae) in the mosquito *Culex renatoi* (Diptera: Culicidae) and the copepod *Paracyclops fimbriatus fimbriatus* (Copepoda: Cyclopidae). J. Euk. Microbiol. 47, 575–580.
Micieli, M.V., Garcia, J.J., Becnel, J.J., 2000b. Horizontal transmission of *Amblyospora albifasciati* Garcia and Becnel, 1994 (Microsporidia: Amblyosporidae) to a copepod intermediate host and the neotropical mosquito, *Aedes albifasciatus* (Macquart, 1837). J. Invertebr. Pathol. 75, 76–83.
Molaei, G., Farajollahi, A., Scott, J.J., Gaugler, R., Andreadis, T.G., 2009. Human bloodfeeding by the recently introduced mosquito, *Aedes japonicus japonicus*, and public health implications. J. Am. Mosq. Control Assoc. 25, 210–214.
Peyton, E.L., Cambell, S.R., Candeletti, T.M., Romanowski, M., Crans, W.J., 1999. *Aedes* (*Finlaya*) *japonicus japonicus* (Theobald), a new introduction into the United States. J. Am. Mosq. Control Assoc. 15, 238–241.
Prenter, J., MacNeil, C., Dick, J.T.A., Dunn, A.M., 2004. Roles of parasites in animal invasions. Trends Ecol. Evol. 19, 385–390.
Reeves, W.K., Korecki, J.A., 2004. *Ochlerotatus japonicus japonicus* (Theobald) (Diptera: Culicidae), a new invasive mosquito for Georgia and South Carolina. Proc. Entomol. Soc. Wash. 106, 233–234.
Roberts, D.W., Panter, C., 1985. Fungi other than *Coelomomyces* and *Lagenidum*. In: Chapman, H.C., Barr, A.R., Laird, M., Weidhas, D.E. (Eds.), Biological Control of Mosquitoes. American Mosquito control Association, Inc., Fresno, CA, pp. 99–109.
Sardelis, M.R., Dohm, D.J., Pagac, B., Andre, R.G., Turell, M.J., 2002a. Experimental transmission of eastern equine encephalitis virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). J. Med. Entomol. 39, 480–484.
Sardelis, M.R., Turell, M.J., Andre, R.G., 2002b. Laboratory transmission of La Crosse virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). J. Med. Entomol. 39, 635–639.
Sardelis, M.R., Turell, M.J., Andre, R.G., 2003. Experimental transmission of St. Louis encephalitis virus by *Ochlerotatus j. japonicus*. J. Am. Mosq. Control Assoc. 19, 159–162.
Schaffner, F., Chouin, S., Guilloteau, J., 2003. First record of *Ochlerotatus* (*Finlaya*)*japonicus japonicus* (Theobald, 1901) in metropolitan France. J. Am. Mosq. Control Assoc. 19, 1–5.
Schaffner, F., Kaufmann, C., Hegglin, D., Mathis, A., 2009. The invasive mosquito *Aedes japonicus* in Central Europe. Med. Vet. Entomol. 23, 448–451.
Simakova, A.V., Andreadis, T.G., Vossbrinck, C.R., 2008. Molecular and ultrastructural characterization of *Andreanna caspii* n. gen., n. sp. (Microsporida: Amlyosporidae), a parasite of *Ochlerotatus caspius* (Diptera: Culicidae). J. Invertebr. Pathol. 99, 302–311.
Sweeney, A.W., 1981. An undescribed species of *Smittium* (Trichomycetes) pathogenic to mosquito larvae in Australia. Trans. Br. Mycol. Soc. 77, 55–60.
Sweeney, A.W., Doggett, S.L., Piper, R.G., 1993. Life cycle of a new species of *Duboscqia* (Microsporida: Thelohaniidae) infecting the mosquito *Anopheles hilli* and an intermediate copepod host, *Apocyclops dengizicus*. J. Invertebr. Pathol. 62, 137–146.
Swofford, D.L., 1998. PAUP* Phylogenetic Analysis Using Parsimony and Other Methods. V. 4.0 Beta. Sinauer associates, Sunderland, MA. <http://paup.csit.fsu.edu/Cmd_ref_v2.pdf>.
Takaoka, H., 1982. Microsporidian infection of *Aedes japonicus* larvae in Japan. Jpn. J. Sanit. Zool. 33, 71–72.
Takaoka, H., Baba, M., Aoki, C., Ogata, K., 1986. Natural infections of larvae of two *Aedes* species with microsporidians and ciliates at the foot of Mt. Sobo in Oita Prefecture. Jpn. J. Sanit. Zool. 37, 189.
Takashima, I., Rosen, L., 1989. Horizontal and vertical transmission of Japanese encephalitis virus by *Aedes japonicus* (Diptera: Culicidae). J. Med. Entomol. 26, 454–458.
Thielman, A., Hunter, F.F., 2006. Establishment of *Ochlerotatus japonicus* (Diptera: Culicidae) in Ontario, Canada. J. Med. Entomol. 43, 138–142.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.
Torchin, M.E., Lafferty, K.D., Dobson, A.P., McKenzie, V.J., Kuris, A.M., 2003. Introduced species and their missing parasites. Nature 421, 628–630.
Turell, M.J., O'Guinn, M.L., Dohm, D.J., Jones, J.W., 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J. Med. Entomol. 38, 130–134.
Vavra, J., Lukes, J., 2013. Microsporidia and the art of living together. Adv. Parasitol. 82, 253–319.
Versteirt, V., Schaffner, F., Garros, C., Dekoninck, W., Coosemans, M., Van Bortel, W., 2009. Introduction and establishment of the exotic mosquito species *Aedes japonicus japonicus* (Diptera: Culicidae) in Belgium. J. Med. Entomol. 46, 1464–1467.
Vossbrinck, C.R., Debrunner-Vossbrinck, B.A., 2005. Molecular phylogeny of the microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitol. 52, 131–142.
Williges, E.A., Farajollahi, A., Scott, J.J., McCuiston, L.J., Crans, W.J., Gaugler, R., 2008. Laboratory colonization of *Aedes japonicus japonicus*. J. Am. Mosq. Control Assoc. 2, 591–593.