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Short Communication

The phylogenetic position of *Ovavesicula popilliae* (Microsporidia) and its relationship to *Antonospora* and *Paranosema* based on small subunit rDNA analysis

Charles R. Vossbrinck *, Theodore G. Andreadis

The Connecticut Agricultural Experiment Station, 123 Huntington Street, P.O. Box 1106, New Haven, CT 06504, USA

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Abstract

Comparative small subunit rDNA sequence analyses, indicate that *Ovavesicula popilliae*, a microsporidian parasite of the Japanese beetle, *Popillia japonica*, represents a distant sister group to *Paranosema* and *Antonospora*. These three genera represent a second major group (the *Nosema*/*Vairimorpha* clade representing the first) of Microsopridia which infect terrestrial insects, suggesting independent origins for both groups. Phylogenetic analyses of *Ovavesicula* and other Microsporidia having a multi-sporous sporogony reveal that this condition is found in several unrelated taxa implying either that multi-sporous sporogony is the ancestral condition for Microsporidia or that it has multiple origins.

Keywords: *Ovavesicula*; *Popillia japonica*; *Paranosema*; Phylogeny; Microsporidia

1. Introduction

Ovavesicula popilliae is a monotypic microsporidian parasite of the Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae) (Andreadis and Hanula, 1987). It is one of six microsporidian species described from scarab beetles (see Hanula and Andreadis, 1992 for list), and is the only microsporidium known from this host. *Ovavesicula popilliae* has been found in populations of *P. japonica* in the northeastern (Connecticut) (Hanula and Andreadis, 1988; Hanula 1990) and the mid-western (Michigan) (Cappaert and Smitley, 2002) United States but has not been reported from hosts in their natural range. The parasite primarily infects the Malphigian tubules, but will occasionally produce systemic infections involving the fat body, epidermis, trachea, oenocytes and pericardial cells in heavily infected larvae and transtadially infected adults (Andreadis and Hanula, 1987; Hanula and Andreadis, 1990). *Ovavesicula popilliae* produces no measurable acute pathology, and typically elicits an intense inflammatory response in the host with accompanying melanization of the pericardium.

Ovavesicula popilliae is one of nine microsporidian taxa (*Baculea*, *Cystosporogenes*, *Endoreticulatus*, *Glugea*, *Ovavesicula*, *Pleistophora*, *Polydysprenia*, *Pseudopleistophora*, and *Vavraia*) that undergo multi-sporous sporogony from a single sporont within a sporophorous vesicle and produce a variable number of spores (usually more than eight). Despite these developmental similarities, *O. popilliae* exhibits several developmental and ultrastructural features that collectively distinguish it from these other multi-sporous "Pleistophora-like" Microsporidia. These features include: (1) diplokaryotic meronts that divide by binary fission; (2) sporonts with unpaired nuclei that develop within a unique, persistent sporophorous vesicle composed of a thick two-layered wall with distinct knob-like protuberances; and (3) synchronous nuclear division (karyokinesis) within the sporogonial plasmodium prior to cytokinesis resulting in

* Corresponding author. Fax: +1 203 974 8502.
E-mail address: charles.vossbrinck@po.state.ct.us (C.R. Vossbrinck).

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the formation of 32 uninucleate spores (Andreadis and Hanula, 1987). Based on these unique characters and fundamental differences in development in comparison to other multi-sporous genera, the creation of a new genus, *Ovavesicula* was proposed with *O. popilliae* as the type species.

Small subunit rDNA sequence data have revealed that some of the developmental features and ultrastructural character states used to designate microsporidian taxa are the result of convergent evolution. For example, the character of producing octospores which separates the *Vairimorpha* species from the *Nosema* species seems to be changing rapidly. As a result, phylogenetic analysis shows a mixing of these two genera with some *Nosema* species (no octospores present) being more closely related to the *Vairimorpha necatrix* group and *Vairimorpha cheracis* being allied with *Nosema bombycis* (Vossbrinck and Debrunner-Vossbrinck, 2005). Therefore, taxa which were thought to be very different taxonomically based on their development and ultrastructural characteristics are, based on rDNA analysis, very similar. At the same time, differences in development, morphology and ultrastructure can suggest possible taxonomic lines. As a result, a number of studies with Microsporidia are now including both ultrastructural and comparative rDNA sequence characters (Maddox et al., 1999; Fries et al., 1999; Andreadis and Vossbrinck, 2002; Sokolova et al., 2003; Vavra et al., 2006) and eventually with the help of phylogenetic analysis, the pattern of ultrastructural changes over evolutionary time will be determined. In this investigation we examine the small subunit rDNA sequence of *O. popilliae* and determine its phylogenetic placement among other multi-sporous Microsporidia.

2. Materials and methods

Spores of *O. popilliae* were obtained from adult Japanese beetles (*Popillia japonica*) that were collected using commercially available pheromone traps (Safer® Japanese Beetle Trap Lure) placed in a suburban location in Wallingford, Connecticut USA. Beetles were collected from the pheromone trap, dissected, and the Malphigian tubules examined for Microsporidia. Infected Malphigian tubules appeared distended and whitish in color. Tubules were homogenized, centrifuged in 50% Percoll (purchased from Sigma–Aldrich) and the spore pellet was collected. The pellet was re-suspended in 500 µl of distilled water and centrifuged again to remove any residual Percoll.

2.1. DNA amplification, sequencing and phylogenetic analysis

Purified spores were broken open by beating in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) in 150 µl STE buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 8.0) and then heated to 95 °C for 5 min. One to five microliters of the STE-ruptured spore solution was removed and used in a standard PCR (94 °C for 3 min, fol-

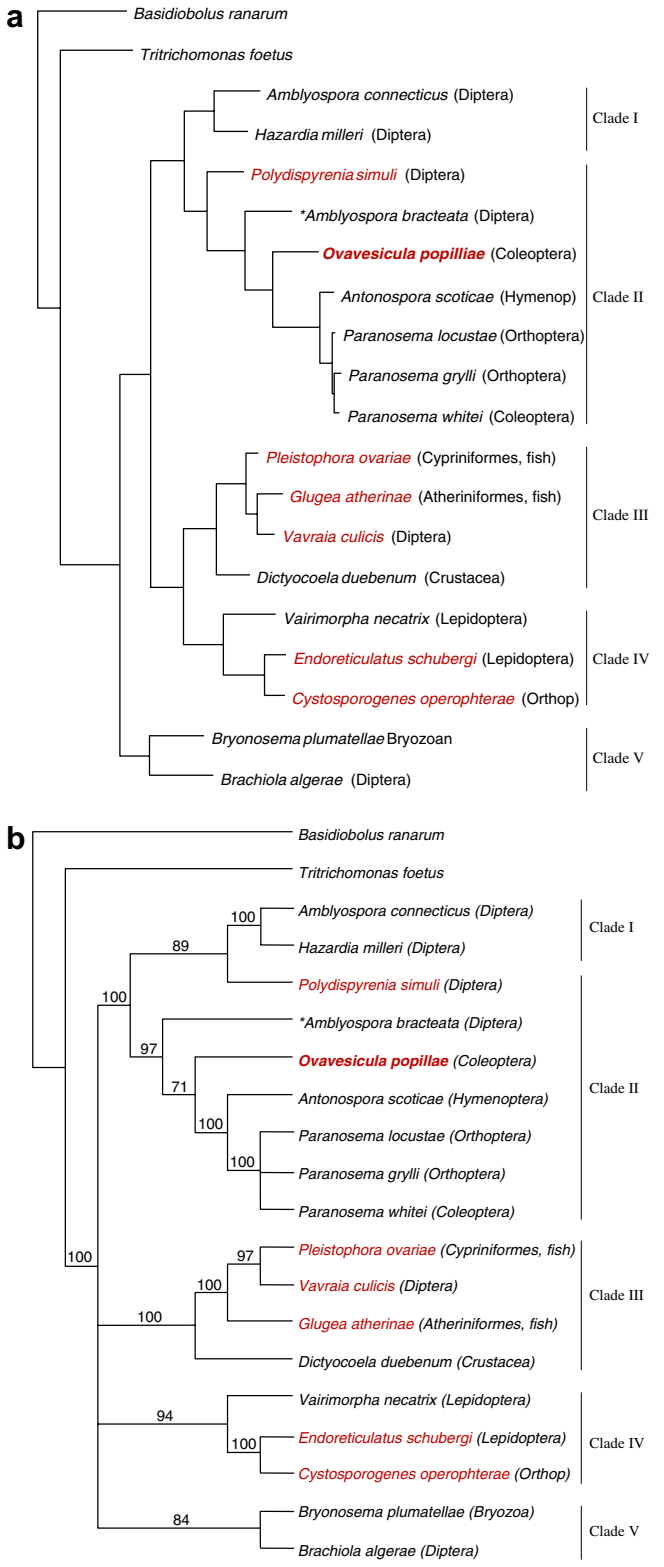

Fig. 1. Phylogenetic trees showing the relationship of *Ovavesicula popilliae* to 17 Microsporidia with a parabasalid flagellate (*Tritrichomonas foetus*) and a fungal (*Basidiobolus ranarum*) outgroup. The host order for each microsporidian species is indicated in parentheses. (a) Results of Maximum Parsimony analysis showing the single shortest tree; (b) bootstrap analysis based on Neighbor Joining analysis (1000 replicates). Taxa in red indicate those with a multi-sporous sporogony. **Amblyspora bracteata* is not a true representative of the genus *Amblyospora*.

Table 1
An uncorrected ("p") distance matrix generated from the sequence data from *Ovavesicula popilliae* and six species of Microsporidia

Microsporidia	1	2	3	4	5	6
1. *Amblyospora connecticus*	—					
2. *Vairimorpha necatrix*	0.346	—				
3. *Ovavesicula popilliae*	0.380	0.401	—			
4. *Antonospora scoticae*	0.338	0.379	0.280	—		
5. *Paranosema locustae*	0.349	0.399	0.276	0.077	—	
6. *Paranosema grylli*	0.343	0.391	0.277	0.083	0.032	—
7. *Paranosema whitei*	0.343	0.392	0.271	0.078	0.030	0.031

lowed by 35 cycles of 94 °C for 45 s, 45 °C for 30 s, and 72 °C for 90 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 sequencing. Sequencing was done at the Keck Biotechnology Resource Laboratory at Yale University with the following microsporidian primers: 18f, CACCAGGTTG ATTCTGCC; SS350f, CCAAGGA(T/C)GGCAGCAGG CGCGAAA; 350r, TTTCGCGCCTGCTGCC(G/A)TC CTTG; SS530f, GTGCCAGC(C/A)GCCGCGG; SS530r, CCGCGG(T/G)GCTGGCAC; 1047r, AACGGCCATG CACCAC; 1061f, GGTGGTGCATGGCCG; and 1492r, GGTTACCTTGTTACGACTT.

Sequences were obtained from the NCBI GenBank database (for accession numbers see Vossbrinck and Debrunner-Vossbrinck, 2005) and were aligned using the Clustal X program (Thompson et al., 1997). No portions of the alignment were changed or eliminated. We selected *Tritrichomonas foetus* as the eukaryotic outgroup. It has been well established, based on both genotypic and phenotypic characters, that *T. foetus* is not a member of the microsporidian clade. *Basidiobolus ranarum* (Zygomycota) was included as a second outgroup because Microsporidia have been established as derived fungi (Edlind et al., 1996; Keeling and Doolittle, 1996). Aligned sequences were analyzed by Maximum Parsimony 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.

3. Results and discussion

DNA sequencing resulted in a 1393 nucleotide small subunit rDNA sequence (GenBank accession number EF564602). It is clear (Fig. 1) that *Ovavesicula* is the sister group to the *Antonospora*/*Paranosema* clade. Percent sequence differences among *Paranosema* species are in the range of 3% and differences between *Antonospora* and *Paranosema* species are 7–8%, while differences between *O. popilliae* and *Paranosema grylli* and *O. popilliae* and *Antonospora scoticae* are 27% and 28%, respectively (see Table 1). *Ovavesicula* therefore represents a very distant sister group to *Paranosema* and *Antonospora* in agreement with the original description based on ultrastructural and developmental characters (Andreadis and Hanula, 1987). Both *Paranosema* and *Antonospora* are morphologically and developmentally distinct from *Ovavesicula*. They are diplokaryotic throughout most of the life cycle, exhibit disporous sporogony, and produce ovocylindrical binucleate spores (Fries et al., 1999; Sokolova et al., 2005).

Ovavesicula along with *Antonospora* and *Paranosema* are parasites of terrestrial insects but are very distantly related (Clade II in Fig. 1) to the Microsporidia of the *Nosema*/*Vairimorpha* clade (Clade IV in Fig. 1). This would imply that these two groups of Microsporidia, which infect terrestrial hosts, have different terrestrial origins and probably evolved from parasites of aquatic hosts.

Fig. 1 also shows 7 genera that have a multi-sporous sporogony coming from 4 of the 5 major clades of Microsporidia (*Polydispyrenia* = Group I, *Ovavesicula* = Group II, *Pleistophora*, *Vavraia* and *Glugea* = Group III, and *Cystosporogenes* and *Endoreticulatus* = Group IV). This would suggest that either this form of sporogony is a plesiomorphic character state (found in the ancestral Microsporidia) and that other groups have lost this character state or, that the multi-sporous condition is polyphyletic in nature and has arisen independently several times.

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