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Short Communication

Occurrence of *Nosema oryzaephili* in *Cryptolestes ferrugineus* and transfer to the genus *Paranosema*

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ABSTRACT

A microsporidium that closely resembles *Paranosema* species at the level of the light microscope was isolated from the rusty grain beetle, *Cryptolestes ferrugineus*. It's identity as *Nosema oryzaephili* (originally described from *Oryzaephilus surinamensis*) was confirmed by comparison with a known isolate of *N. oryzaephili* based on spore size, small subunit rDNA sequence, and relative infectivity to *O. surinamensis*, *Tribolium castaneum*, and *Ephestia kuehniella*. Phylogenetic analysis of the small subunit rDNA indicates clearly that this species belongs in the genus *Paranosema* and thus the designation *Paranosema oryzaephili* (Burges, Canning and Hurst) is proposed. In spite of the abundance, economic importance, and worldwide distribution of *C. ferrugineus*, this is the first report of a microsporidial infection in this species. This is also the first report of *P. oryzaephili* in the new world.

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1. Introduction

The genus *Paranosema* was erected to include certain species that were formerly described in the genus *Nosema* but were distinct from the clade that included *Nosema bombycis* (the type species for the genus *Nosema*) based on phylogenetic analysis of the small subunit ribosomal DNA (ssrDNA) (Baker et al., 1994; Sokolova et al., 2003. There are currently three species assigned to the genus *Paranosema*: *Paranosema grylli* (Sokolova et al., 1994, 2003) from the cricket, *Gryllus bimaculatus* De Geer; *Paranosema locustae* (Canning, 1953), a pathogen of grasshoppers; and *Paranosema whitei* (Weiser, 1953), described from the tenebrionid, *Tribolium castaneum* (Herbst).

Samples from two diseased laboratory colonies of the laemophloeid beetle, *Cryptolestes ferrugineus* (Stephens), were received from Oklahoma State University. Microscopic examination revealed that many of the beetles from these colonies were infected with a *Nosema*-like microsporidium. There are no reports, to our knowledge, of Microsporidia infecting *C. ferrugineus* despite the fact that the beetle has been extensively studied, occurs throughout the world, infests many commodities, and is the most abundant insect in northern grain stores (Bishop, 1959). We conducted bioassays on several granivorous insects to clarify the physiological host range within the guild and to confirm its identity. When infectivity for *T. castaneum* and *Oryzaephilus surinamensis* (L.) were confirmed, we obtained spore measurements and sequenced the ssrDNA to establish the identity and taxonomic position of this pathogen. We report here the first case of a microsporidium infecting *C. ferrugineus* and transfer the generic designation of *Nosema oryzaephili* to create the new combination *Paranosema oryzaephili*.

2. Materials and methods

2.1. Assays

Red flour beetles, *T. castaneum*; rusty grain beetles, *C. ferrugineus*; sawtoothed grain beetles, *O. surinamensis*; and Mediterranean flour moths, *Ephestia kuehniella* Zeller, were obtained from colonies of eastern Kansas origin and maintained at the USDA Center for Grain and Animal Health Research, Manhattan, KS. Our microsporidial isolate from *C. ferrugineus* was maintained and produced in larval *C. ferrugineus*. To test host range of this isolate, spores were mixed into a diet of wheat flour with 4% brewer's yeast and 4% wheat germ, and larvae of each tested insect species were exposed to spores 3–4 days after hatching. The larvae were placed in individual assay tray wells (C-D International Inc., Pitman, NJ) with 50 mg of diet containing 0, 10^4, 10^5, or 10^6 spores/g. Assays were incubated for 21 days at 30 °C and 75% RH in continuous darkness. At the end of the incubation period, squashes of all larvae were examined for the presence of spores with phase contrast microscopy at 400×. There were 32 larvae per replicate. There were three replicates for the beetles carried out at separate times with separate cohorts of insects. For *E. kuehniella*, the assay was not repeated, and the doses were 0, 10^5 or 10^6 spores/g. The data for beetle

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assays with the new isolate were subjected to ANOVA using SigmaStat 3.1 (Systat Software, Richmond, CA.)

For comparative purposes we assayed the *N. orzyaephili* isolate obtained from its type host, *O. surinamensis*, against *C. ferrugineus* to see if it would infect this host in a similar manner. Rusty grain beetle larvae were exposed as described above in diet containing 0, 10^4, 10^5 or 10^6 spores/g. Only one trial was run.

Finally, we assayed *P. whitei* at 10^6 spores/g against *C. ferrugineus* to examine its infectivity to this species and against a known host, *T. castaneum*.

The *P. whitei* isolate that was used for these assays was obtained from the Illinois Natural History Survey collection of Dr. Leellen Solter and is the isolate that was used to obtain the published ssrDNA sequence for *P. whitei* (Sokolova et al., 2003).

2.2. Spore measurements

Fresh spores in squashed *T. castaneum* larvae were measured with an Image-Splitting Eyepiece (Vickers Instruments, Ltd.) and compared with one hundred spores each of *P. whitei* and our new isolate from *C. ferrugineus*. Methanol-fixed, Giemsa-stained merонts were also measured.

2.3. DNA isolation

DNA isolation, gene amplification, and DNA sequencing were performed as described elsewhere (Vossbrinck et al., 1998). Spores were purified using a 50% Percoll cushion in a 1.5 ml Eppendorf microcentrifuge tube. DNA was liberated from spores by bead beating in STE buffer followed by heating at 95 °C for 5 min. A 3 μl sample was amplified using Primers 18f and 1492r using the Qiagen Taq PCR Kit following the manufacturer's instructions. Amplified DNA was purified on QIAquick PCR purification spin columns (Qiagen, Valencia, CA) and submitted for sequencing using previously published primers (Vossbrinck et al., 1998; Weiss and Vossbrinck, 1998, 1999). In addition to sequencing the isolate from *C. ferrugineus* we obtained and sequenced an isolate from *O. surinamensis*, the type host for *P. oryzaephili*, to assure that sequences were identical. They did.

Fourteen species were selected for phylogenetic analysis. *Basidiobolus ranarum* was selected as a fungal (Zygomycetes) outgroup. *Loma salmonae*, *Amblyospora connecticus*, *Bryonosema plumatellae* and *Octosporea bayeri* were included as representatives of distantly related microsporidial taxa. In addition *N. bombycis* and *Vairimorpha necatrix* were included as part of the *Nosema/Vairimporha* clade. *Amblyospora bracteata*, *Ovavesicula popillae* and *Antonospora scoticae* were added because of their inclusion with *Paranosema* in Clade II (Vossbrinck and Andreadis, 2007).

The alignment was created by SequentiX (http://www.sequentix.de) based on rDNA secondary structure. Maximum parsimony and neighbor-joining bootstrap analysis were conducted using PAUP version 4.0b10 for Macintosh computer. All characters were type unordered and all characters had equal weight. Maximum likelihood analysis was accomplished on line at the Methodes et Algorithmes pour la Bio-informatique site (http://www.phylogeny.fr) using the same alignment at the default settings.

3. Results and discussion

In this study we compared a new isolate to two morphologically similar microsporidial species from known granivorous insects, namely *P. whitei* and *N. oryzaephili*. Host range overlap and morphological similarity of *P. whitei* and *N. oryzaephili* from previous reports make these two species difficult to distinguish. Burges et al. (1971) reported an LC_{50} of 2.6×10^5 spores/g for *O. surinamensis* exposed to *N. oryzaephili*, but low pathogenicity for *T. castaneum*. The authors deemed this low virulence of *N. oryzaephili* for *T. castaneum* a distinguishing characteristic since *P. whitei* is highly virulent in *Tribolium* spp. Conversely, Milner (1973) reported that *P. whitei* had similar LD_{50} rates for *O. surinamensis* and *T. castaneum*.

Of the beetle species tested in this study, the *C. ferrugineus* isolate was most infectious to the host from which it was isolated (Table 1). At a dose of 10^4 spores/g, the infection rate of *C. ferrugineus* was significantly higher than that of *T. castaneum* and *O. surinamensis* ($F = 8.2$; $df = 2.6$; $P = 0.02$), but the infection rates of *T. castaneum* and *O. surinamensis* did not differ significantly. At a dose of 10^5 spores/g, the mean infection rate of *C. ferrugineus* was significantly greater than that of *O. surinamensis*, and the infection rate of *O. surinamensis* was significantly greater than that of *T. castaneum* ($F = 37.7$; $df = 2.6$; $P < 0.01$). Our new isolate had a greater infection rate in *O. surinamensis* than *T. castaneum* which is consistent with previous studies on *N. oryzaephili*. The fact that the new isolate was more infectious for *C. ferrugineus* than for even its type host may be a reflection of the randomness of pathogen observations and isolation. The type host may not be the predominant host in nature.

The exposure of 32 individual neonate *E. kuhniella* at a concentration of 10^5 *N. oryzaephili* spores/g resulted in 11 of 32 larvae becoming infected. At a concentration of 10^6 spores/g, 27 larvae became infected with 22 dead after the 21 day incubation period. These results are consistent with the findings of Burges et al. (1971), who reported infection of 31% of 14-day-old *E. kuehniella* larvae that were exposed to *N. oryzaephili* at 10^7 spores/g of diet.

Exposures to 10^6 spores/g of *P. whitei* resulted in 89% infection of *T. castaneum* showing that, like *N. oryzaephili*, *P. whitei* can infect *T. castaneum*. However, unlike both *P. oryzaephili* isolates, *P. whitei* cannot infect *C. ferrugineus* at even the highest exposure levels used. Infectivity to *C. ferrugineus* therefore is a good means for distinguishing between *P. whitei* and *N. oryzaephili*.

We show similar rates of infection between our isolate and the putative *N. oryzaephili* previously isolated from *O. surinamensis* (Table 1). In addition, our observations of stages in the fat body such as the presence of both diplokarya and individual nuclei and the common appearance of tetranucleate meronts (Fig. 1) are very similar to those described by Burges et al. (1971). Our measurements of fresh spores under phase contrast illumination (4.5 (SD = 0.23) × 2.9 (SD = 0.17) μm) and methanol-fixed binucleate meronts (5.2 (SD = 0.73) × 4.2 (SD = 0.66) μm) fall within the ranges of those reported for *N. oryzaephili* (Burges et al., 1971).

Table 1
Mean percentage infection (SD) of host beetle larvae after three weeks of exposure to microsporidia of granivorous insects.

	Dosage (spores/g of diet)		
	10^4	10^5	10^6
Nosema oryzaephili from Cryptolestes ferrugineus			
C. ferrugineus	77 ± 25a	99 ± 2a	99 ± 2a
Oryzaephilus surinamensis	36 ± 22b	69 ± 6b	81 ± 6a
Tribolium castaneum	13 ± 1b	51 ± 9c	83 ± 21a
Ephestia kuehniella	–	34	84
N. oryzaephili from O. surinamensis			
C. ferrugineus	54	89	100
Paranosema whitei			
T. castaneum	–	–	89
C. ferrugineus	–	–	0

Infection percentages within column followed by the same letter are not significantly different by Fisher LSD test ($\alpha = 0.05$). Percentages lacking letters are from unreplicated assays.

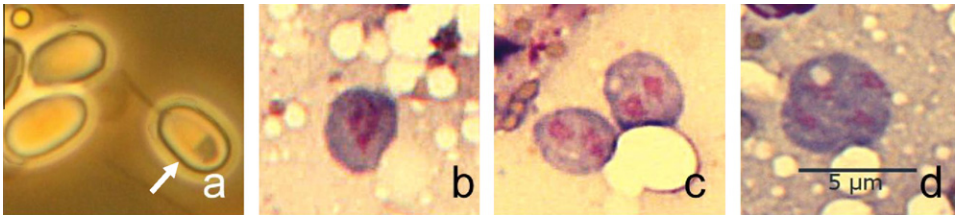

Fig. 1. Light micrographs of *Paranosema oryzaephili*. (a) Intact and germinating (arrow) fresh spores under phase contrast. (b) Diplokaryotic meront stained with Giemsa. (c) Binucleate meronts with monokaryotic nuclei, Giemsa-stained. (d) Tetranucleate meront, Giemsa-stained.

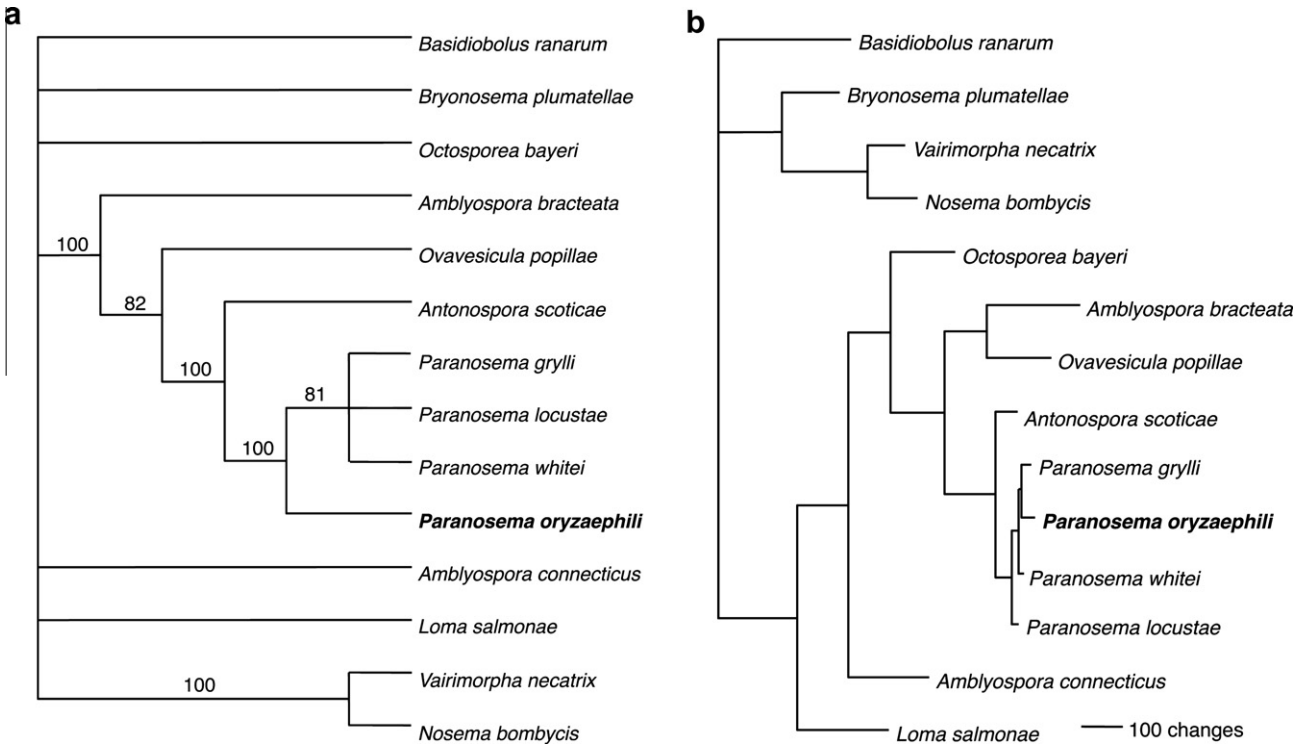

Fig. 2. Bootstrap analysis using the neighbor joining analysis (a) and Phylogram of the maximum parsimony analysis (b). *Basidiobolus ranarum* (Class Zygomycetes) is included as a fungal outgroup. Representatives of the major microsporidial taxa have been added to show the wide separation between *Paranosema* species and the *Nosema*/*Vairimorpha* clade.

Finally, ssrDNA sequences for both the *O. surinamensis* and *C. ferrugineus* isolates were identical lending strong support for the identity of this isolate as *N. oryzaephili*.

Small subunit rDNA analysis is the basis for establishment of the genus *Paranosema* (Sokolova et al., 2003). The SSU rDNA sequence obtained from the isolate under investigation (GenBank accession number HM002483) contained 1362 nucleotides. Our analysis showed that *N. oryzaephili* falls within Clade II of Vossbrinck and Debrunner-Vossbrinck (2005). Maximum likelihood and neighbor joining analysis place our *P. oryzaephili* as a sister taxon to the remaining *Paranosema* species (Fig. 2A). This reflects the fact that *P. oryzaephili* showed more differences from the other *Paranosema* species than they did from each other. Maximum parsimony analysis however indicates that *P. oryzaephili* is most closely related to *P. grylli* (Fig. 2B). In either case, these four species group together with a neighbor-joining bootstrap value of 100, have a maximum likelihood value of 1 and have a high sequence similarity (uncorrected "*P*" value of .041 or less) indicating that this isolate is clearly a *Paranosema* species.

In spite of its abundance and world-wide distribution, this is the first report of *C. ferrugineus* infection with a microsporidium. It is also the first report of *N. oryzaephili* from the new world.

The new combination *P. oryzaephili* (Burges, Canning & Hurst) is proposed.

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