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Short Communication

A new isolate of *Nosema* sp. (Microsporidia, Nosematidae) from *Phyllobrotica armata* Baly (Coleoptera, Chrysomelidae) from China

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ABSTRACT

We studied the spore morphology and molecular systematics of a novel microsporidian isolate from *Phyllobrotica armata* Baly collected in China. The spores were long-oval and measured 4.7 × 2.6 μm on fresh smears. Ultrastructure of the spores was characteristic for the genus *Nosema*: 13–14 polar filament coils, posterior vacuole, and a diplokaryon. The complete rRNA gene sequence of the isolate was 4308 bp long. The organization of the rRNA gene was 5′-LSU rRNA-ITS-SSU rRNA-IGS-5S-3′, which corresponds to that of the *Nosema* species. Phylogenetic analysis based on the rRNA gene sequence indicated that this isolate, designated as *Nosema* sp. PA, is closely related to *Nosema bombycis* and is correctly assigned to the "true" *Nosema* group.

1. Introduction

Microsporidia are minute (diameter, 1–40 μm) unicellular eukaryotes that are specialized obligate intracellular parasites of other eukaryotes. These organisms are emerging pathogens and are serious pests in sericulture, apiculture, and fisheries (Wittner and Weiss, 1999). Of the 1300 microsporidian species described in the literature so far (Corradi and Keeling, 2009), at least 200 belong to the genus *Nosema* (Sprague, 1982). Moreover, the most common microsporidia that infect Lepidoptera are members of the genus *Nosema* (Tsai et al., 2003). Baker et al. (1994) suggested that genus *Nosema* is a heterogeneous assemblage of more or less related genera. This genus includes a group of "true" *Nosema* species, as defined by the type species *Nosema bombycis*, and another group comprising species of *Vairimorpha* (type species *Vairimorpha necatrix*) and some other *Nosema* spp.

Among the many diseases that affect the silkworm *Bombyx mori*, pebrine—caused by the microsporidium *N. bombycis*—is the most devastating. This disease results in severe losses in the silk cocoon crop and it is transmitted to the next generation transovarially. However, some microsporidia isolated from other wild insect species can also cause pebrine in silkworm (Kishore et al., 1994; Bhat et al., 2009). We isolated a novel microsporidium from a mulberry leaf beetle, *Phyllobrotica armata* Baly. This isolate appeared to be morphologically and genetically similar to the species of the *N. bombycis* group. A preliminary study revealed that this isolate (designated as *Nosema* sp. PA) not only heavily infected the silk glands, but also spread to other tissues, including the alimentary canal, malpighian tubules, muscle, fat bodies, epithelium, and gonads. *Nosema* sp. PA can infect silkworm and cause pebrine; however, the pathogenicity of this isolate to the silkworm is far lower than that of *N. bombycis* (unpublished results). This paper focuses on the characterization of *Nosema* sp. PA and its phylogenetic relationships with other microsporidia.

2. Materials and methods

2.1. Purification of spores

Infected beetles were collected and homogenized in sterile water. The homogenates were filtered through four layers of cheesecloth and centrifuged at 3000×*g* for 15 min. The pellets were resuspended in sterile water, and the spores were purified by Percoll gradient centrifugation using 90% Percoll at 15,000×*g* for 40 min. The spore band was collected and washed several times with sterile water (Tsai et al., 2003; Johny et al., 2006). The purified spores ($n = 50$) were measured under a light microscope (IL/Leica Microsystems, Inc., Deerfield) with an ocular micrometer and photographed with the Microscope USB Camera.

2.2. Transmission electron microscopy

Electron microscopy was performed as previously described (Choi et al., 2002) with slight modifications. The purified spores

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of *Nosema* sp. PA were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight. Samples were postfixed for 3 h in 1% osmium tetroxide, dehydrated through ascending ethanol series, and embedded in Epon–Araldite. Ultrathin sections were cut by using the Reichert–Jung ULTRACUT E ultramicrotome, stained in methanolic uranyl acetate, and then in lead citrate. The stained grids were observed by using the JEM-1230 (JEOL Ltd.) transmission electron microscope.

2.3. Molecular phylogenetic analysis

Genomic DNA was extracted as previously described (Dong et al., 2010). The PCR reactions, primer sets, and sequencing methods used were also as described in a previous paper (Zhu et al., 2010). Sequences were obtained from the NCBI GeneBank database and were aligned using the ClustalX 1.83 program. The rRNA gene sequences of *Encephalitozoon cuniculi* was used as the outgroup. Phylogenetic trees were constructed with nucleotide sequences by using the MEGA version 4 (Tamura et al., 2007). The evolutionary history was inferred by using the maximum parsimony (MP) method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown adjacent to the branches. The MP tree was obtained using the close-neighbor-interchange algorithm with search level 3, in which the initial trees were obtained with random addition of sequences (10 replicates). The tree is drawn to scale with branch lengths calculated by using the average pathway method, and were in the units of the number of changes over the whole sequence. All alignment gaps were treated as missing data.

3. Results

3.1. Morphological characteristics

Light microscopy revealed that fresh *Nosema* sp. PA spores were generally long-oval. They had a mean length and mean width of 4.7 μm (SD 0.3) and 2.6 μm (SD 0.2), respectively (Fig. 1A). Electron micrography of a longitudinal section of a mature spore revealed that the spore wall consisted of an electron-dense exospore (thickness, approximately 40 nm) and electron-lucent endospore layer and that the sporoplasm was enclosed by a plasma membrane. The coiled region of the polar tube comprised 13–14 turns, and the diplokaryotic nuclei were slightly separated from each other. A membrane-bound vacuole with amorphous content was located at the posterior end of the spore (Fig. 1B). All the above-mentioned features correspond to the principle characteristics of the genus *Nosema* (Larsson, 1986).

3.2. Analysis of the entire sequence of Nosema sp. PA rRNA

The complete sequence of the *Nosema* sp. PA rRNA gene was 4308 bp long (NCBI GenBank accession No. FJ969508). The gene arrangement from the 5′ end is as follows: the large-subunit gene (LSU rRNA, 2493 bp), internal transcribed spacer (ITS, 186 bp), small-subunit gene (SSU rRNA, 1233 bp), intergenic spacer (IGS, 281 bp), and 5S region (5S, 115 bp).

The sequence identity of the *Nosema* sp. PA rRNA to the rRNA of other selected microsporidian species is shown in Fig. 2A. The LSU, SSU, and 5S regions of the *Nosema* sp. PA rRNA show 94.7–99.9% identity with the corresponding regions in the rRNA of "true" *Nosema* species (*N. bombycis*, *N. spodopterae*, *N. plutellae*, uncultured *Nosema*, *Nosema* sp. PX1, and *N. antheraeae*). The results also suggest that this isolate may be most closely related to *Nosema* sp. PX1, with which it shares 97.8% identity of total rRNA gene (LSU, 99.4%; ITS, 89.7%; SSU, 99.9%; IGS, 82.2%; 5S, 97.4%). The rRNA of the novel isolate also shares high sequence similarity with that of the uncultured *Nosema* (GenBank accession No. EU338534): 97.8% identity of the total rRNA gene and 93.5% of the ITS region. *Nosema* sp. PA has a higher identity at the ITS region to uncultured *Nosema* than that to the other microsporidia.

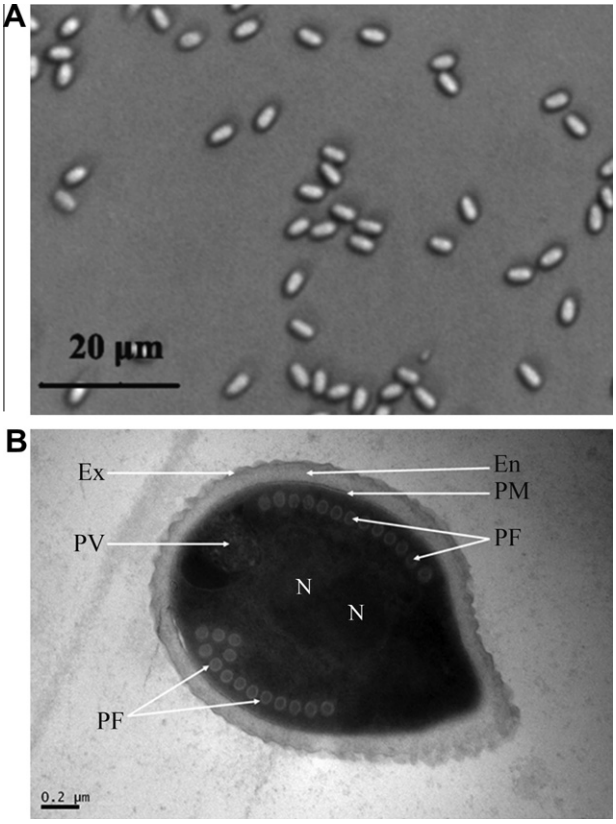

Fig. 1. Micrographs of the *Nosema* sp. PA spores. (A) Light micrograph of the *Nosema* sp. PA spores after Percoll purification. Scale bar = 20 μm. (B) Electron micrograph of a longitudinal section of a *Nosema* sp. PA spore. The nucleus (N), exospore (Ex), endospore (En), plasma membrane (PM), polar filament (PF), and posterior vacuole (PV) are visible. Scale bar = 0.2 μm.

3.3. Molecular phylogeny

Maximum parsimony analysis based on the rRNA and LSU rRNA genes was performed (Fig. 2). In the rRNA-based maximum parsimony analysis (Fig. 2B), *Nosema* sp. PA was found to be most closely related to the uncultured *Nosema* followed by *N. bombycis* and *N. spodopterae*. In the analysis based on LSU rRNA (Fig. 2C), the novel isolate was grouped with *Nosema* sp. PX1, uncultured *Nosema*, *N. bombycis*, and *N. spodopterae*. On the basis of the results of both analyses, *Nosema* sp. PA was assigned to the *N. bombycis* group within the genus *Nosema*.

4. Discussion

The SSU rRNA sequence has been widely used as a molecular marker for estimating relationships among microsporidia; however, Canning et al. (1999) and Tsai et al. (2003) suggested that this highly conserved gene could not be used to distinguish between closely related species. Therefore, other markers were required

A

	Total rRNA	LSU	ITS	SSU	IGS	5S
Nosema bombycis	97.6	99.3	89.3	99.8	82.5	94.7
Nosema spodopterae	97.6	99.4	89.1	99.9	81.2	96.5
Nosema plutellae	96.1	98.4	82.4	98.5	70.7	97.4
uncultured *Nosema*	97.8	99.2	93.5	99.2	82.2	97.4
Nosema sp. PX1	97.8	99.4	89.7	99.9	82.2	97.4
Nosema antheraeae	—	—	83.2	99.4	69.4	95.7
Nosema apis	66.2	78.2	46.9	84.4	—	—
Nosema ceranae	66.2	78.7	63.2	84.7	—	—
Nosema sp. C01	75.3	79.3	69.4	98.3	—	—
Encephalitozoon cuniculi	49.0	63.7	50.0	69.9	—	—

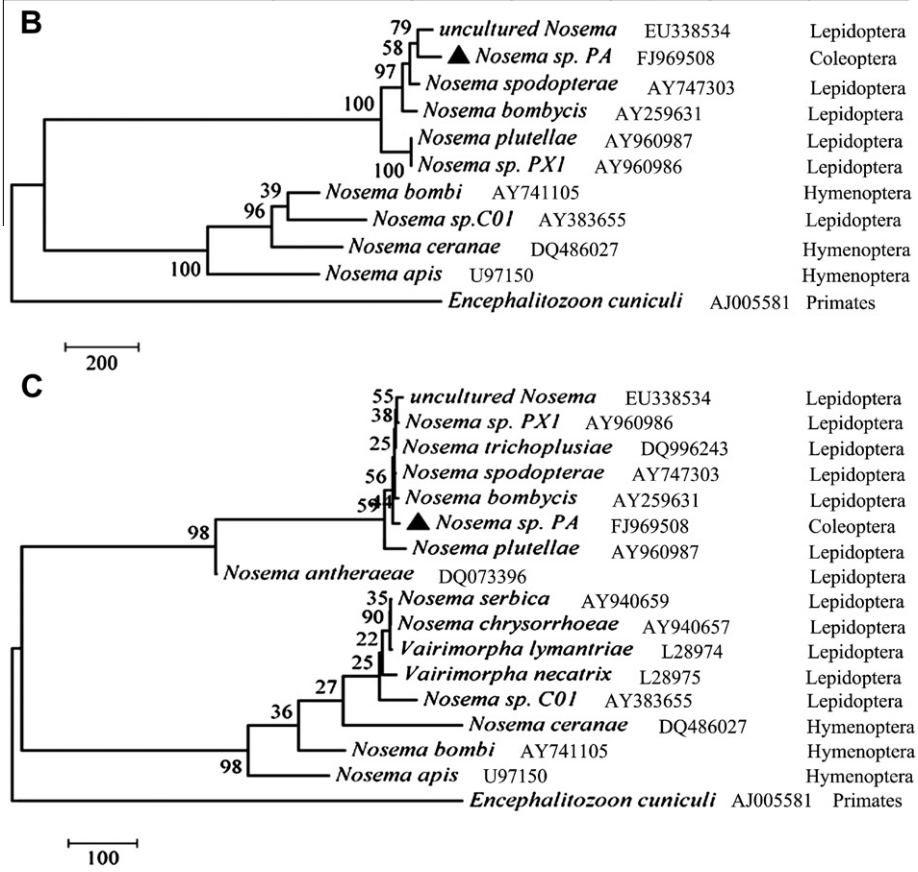

Fig. 2. The rRNA gene identities (%) and phylogenetic analysis of the *Nosema* sp. PA based on the rRNA and LSU rRNA genes. (A) Identities (%) of the various regions of the rRNA gene of *Nosema* sp. PA and other microsporidia species. (B) A phylogenetic tree based on the rRNA sequences. (C) A phylogenetic tree based on the LSU rRNA sequences. Phylogenetic trees were constructed by using the maximum parsimony method. The bootstrap values are indicated at the nodes. The GenBank accession number for each sequence is given adjacent to the corresponding species name.

for analyzing phylogenetic affinities. Tsai et al. (2005) suggested that the microsporidian ITS and LSU rRNA sequences have good potential as informative molecular markers. According to the results of our study, the identity of the rRNA gene sequences of *Nosema* sp. PA with those of the other *Nosema* species is high (Fig. 2A). Phylogenetic analysis (Fig. 2B and C) revealed that this isolate is closely related to the members of the "true" *Nosema* group, which is consistent with the findings of an earlier study (Dong et al., 2010). In the phylogenetic tree based on the LSU gene, which was outlined in this paper, all *Nosema* species that infect Lepidoptera, except the *Nosema* sp. PA isolated from a beetle, belong to the "true" *Nosema* group. The *Vairimorpha* spp. and other *Nosema* spp. form a separate branch in the phylogenetic tree (see also Rao et al., 2004). In other words, the *Nosema* species that infect only Lepidoptera species are more closely related to each other than they are to other *Nosema* species that infect non-lepidopteran hosts. Rao et al. (2004) indicated that the distinct grouping of *Nosema* species that infect only Lepidopteran hosts might be caused by the coevolution of the lepidopteran-infecting *Nosema* species with their host group over time.

The organization of the rRNA gene of *Nosema* sp. PA is 5′-LSU-ITS-SSU-IGS-5S-3′, which is a pattern similar to the rRNA gene of *N. bombycis* (Huang et al., 2004), *N. spodopterae* (Tsai et al., 2005), *N. antheraeae* (Wang et al., 2006), *N. plutellae* (Ku et al., 2007), and uncultured *Nosema* (Tsai et al. 2009). The novel arrangement of the rRNA gene may be an important feature of the "true" *Nosema* group (Tsai et al., 2005; Kyei-Poku et al., 2008). According to the results of the morphological and genetic analyses, we suggest that

the microsporidian *Nosema* sp. PA belongs to the "true" *Nosema* group.

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