

## Molecular characteristics of the alpha- and beta-tubulin genes of *Nosema philosamiae*

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**Abstract:** Microsporidia are intracellular parasites of insects and other higher eukaryotes. The microsporidian *Nosema philosamiae* Talukdar, 1961 was isolated from the eri silkworm, *Philosamia cynthia ricini* Grote. In the present study, alpha- and beta-tubulin genes from *N. philosamiae* were characterized. The identity analysis of nucleotide and amino acid sequences indicated high similarity with species of *Nosema* Nägeli, 1857 *sensu lato* (nucleotide sequences,  $\geq 96.0\%$ ; amino acid sequences,  $\geq 99.0\%$ ). However, the tubulin genes of *N. philosamiae* share low sequence similarity with that of *N. ceranae* Fries, Feng, da Silva, Slemenda et Pieniazek, 1996 (strain BRL01) and a *Nosema/Vairimorpha* species. Phylogenies based on alpha-, beta- and combined alpha- plus beta-tubulin gene sequences showed that *N. philosamiae*, along with the true *Nosema* species, forms a separate clade with a high bootstrap value, with *N. ceranae* BRL01 forming a clade of its own. The results indicated that the alpha- and beta-tubulin sequences may be useful as a diagnostic tool to discriminate the true *Nosema* group from the *Nosema/Vairimorpha* group.

**Keywords:** microsporidia, *Nosema* group, pairwise distance, phylogeny, sequence similarity

This article contains supporting information (Table S1–S5) online at <http://folia.paru.cas.cz/suppl/2013-60-5-411.pdf>

Microsporidia is a large group of organisms characterized by a highly reduced and compact genome. Molecular karyotype analysis and genome sequencing of several microsporidian species indicate that the human parasite *Encephalitozoon intestinalis* Cali, Kotler et Orenstein, 1993 has the smallest known genome among eukaryotes (2.3 Mbp) and that many other species, such as *Nosema locustae* Canning, 1953, *Enterocytozoon bieneusi* Desportes, 1985, *Spraguea lophii* Doflein, 1898, have genomes smaller than 10 Mbp (Biderre et al. 1994, Peyre-taillade et al. 1998, 2011, Katinka et al. 2001). Although analyses involving SSU rRNA (Vossbrinck et al. 1987) and the elongation factors EF-1 $\alpha$  and EF-2 (Kamaishi et al. 1996a, 1996b) place these organisms in one of the most basal eukaryotic lineages, recent molecular phylogenetic studies support the placement of these eukaryotes amongst fungi, with a probable zygomycete ancestor (Lee et al. 2008a, Texier et al. 2010).

The tubulin gene family consists of six distinct, but highly conserved subfamilies that possess alpha-, beta-, gamma-, delta-, epsilon-, and zeta-tubulins, each defined by sequence conservation and a wide distribution among eukaryotes (McKean et al. 2001). Heterodimers of alpha- and beta-tubulin proteins are the major components of microtubules, which in turn are central to the composition of eukaryotic cilia, flagella, mitotic spindles and the cy-

toskeleton. Microsporidians have only alpha-, beta-, and gamma-tubulin genes. Eukaryotic alpha- and beta-tubulin proteins have been extensively studied, because they are the most abundant proteins in eukaryotic cells and their evolution may have paralleled that of the nucleus (McKean et al. 2001). The beta-tubulin gene is fairly conserved, with higher amino acid similarity ( $> 60\%$ ) between the most distantly related lineages (Juuti et al. 2005).

Relationships among the taxa of microsporidians have been inferred primarily from analyses of small subunit rRNA (SSU rRNA) sequences (Vossbrinck and Debrunner-Vossbrinck 2005). However, data on recent phylogenies based on alpha- and beta-tubulin protein-coding genes have enabled to carry out more robust phylogenetic analyses (Keeling et al. 2000, Keeling 2003, Akiyoshi et al. 2007, Lee et al. 2008b, Johny et al. 2009, Haag et al. 2011).

The microsporidium *Nosema philosamiae* Talukdar, 1961 was isolated from the eri silkworm, *Philosamia cynthia ricini* Grote, in Zhenjiang City, Jiangsu Province, China. This species has been the key factor in obstructing the development of sericulture in China. Studies on the complete rRNA genes and morphological characteristics indicate that this species is closely related to the species of ‘true’ *Nosema* (see Zhu et al. 2010).

In the present study, alpha- and beta-tubulin genes of *N. philosamiae* were cloned partially; the nucleotide se-

quences were compared with corresponding sequences of other microsporidian species for which such data were available, especially species of *Nosema* Nägeli, 1857.

## MATERIALS AND METHODS

### Materials and reagents

The spores of *Nosema philosamiae* were isolated from eri silkworms and preserved at the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (SRI-CAAS). LA-Taq enzyme, restriction enzymes, pMD18-T vectors and T4 DNA ligase were purchased from Takara Company (Takara Bio Inc., Shiga-ken, Japan).

### DNA isolation, PCR amplification and sequencing

Genomic DNA was extracted from the microsporidian as described by Dong et al. (2010). The sequences of the primers (5'-TCCGAATTCARGTNGGNAAYGCNGGYTGGGA-3' – forward and 5'-CGCGCCATNCCYTCNCCNACRTACCA-3' – reverse) used for the amplification of the alpha-tubulin gene were from the study by Lee et al. (2008). The primers (5'-GTAG-GAGGAAAGTTCCTGGGAG-3' – forward and 5'-TCCTTCAC-CAGTGTACCACTG-3' – reverse) used for the amplification of beta-tubulin gene were designed using Premier Primer 5.0 software based on the conserved 5' and 3' end regions of beta-tubulin genes of four *Nosema* species, i.e. *N. bombycis* Nägeli, 1857; *N. plutellae* Ku, Wang, Tsai, Tzeng et Wang, 2007; *Nosema* sp. of Tsai, Lo, Soichi et Wang, 2003 (strain PX1); and *N. spodopterae* Tsai, Lo, Soichi et Wang, 2003.

The PCR protocol followed was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles, each comprising heating for 30 sec at 95°C, 30 sec at 50°C, and 1 min 30 sec at 72°C, and final extension at 72°C for 10 min. The amplified products were subjected to 1.0% agarose gel electrophoresis, cloned into pMD18-T vectors (Takara Bio Inc., Shiga-ken, Japan), and then sequenced.

### Data analysis

The length and GC content of partial alpha- and beta-tubulin gene sequences of *N. philosamiae* were analysed using the Editseq program of the DNASTar software package (DNASTAR, Madison, Wisconsin, USA). The identity and pairwise distance of the common sequences of selected microsporidian species were analysed using the MegAlign program (DNASTAR, Madison, Wisconsin, USA). The alpha- and beta-tubulin gene sequences were deduced in terms of amino acid sequences by using the Genetyx software (Software Development, Tokyo, Japan).

### Phylogenetic analysis

The nucleotide sequences obtained in the present study and those for microsporidian species compared, including those for 14 alpha-tubulin genes and 15 beta-tubulin genes, were aligned with the Clustal X 1.83 software (Thompson et al. 1997). Phylogenetic trees were constructed with nucleotide sequences of protein-coding genes using the MEGA 5 software package (Tamura et al. 2011). The evolutionary history was inferred by using the maximum likelihood (ML) and maximum parsimony (MP) methods. The bootstrap consensus tree inferred from 1 000 replicates was used to represent the evolutionary history of the taxa analysed. 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 (1 000 replicates) is shown next to

the branches. First and second codon positions were included, whereas the third position was excluded. *Trachipleistophora hominis* Hollister, Canning, Weidner, Field, Kench et Marriott, 1996 was used as the outgroup.

## RESULTS

### Characterization of tubulin genes

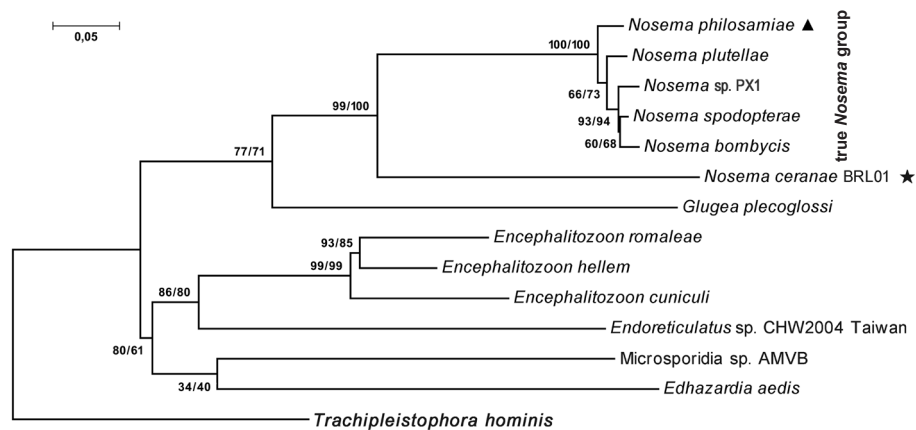
New alpha- and beta-tubulin partial gene sequences from *Nosema philosamiae* have been deposited in GenBank (accession numbers are shown in Table S1). The *N. philosamiae* alpha-tubulin partial gene contained an open reading frame of 1 198 bp with a GC content of 36.5% and shared high sequence similarity with true *Nosema* species (nucleotide sequences, *N. bombycis* – 96.5%, *N. spodopterae* – 96.5%, *N. plutellae* – 97.0%, and *Nosema* sp. PX1 – 96.4%; amino acid sequences, *N. bombycis* – 100%, *N. spodopterae* – 100%, *N. plutellae* – 100% and *Nosema* sp. PX1 – 99.5%) (Tables S2, S3).

However, the alpha-tubulin gene of *N. philosamiae* shares low sequence similarity with that of the *N. ceranae* BRL01 Fries, Feng, da Silva, Slemenda et Pieniazek, 1996, a *Nosema/Vairimorpha* species: 77.8% identity of the nucleotide sequences and 89.2% identity of the amino acid sequences.

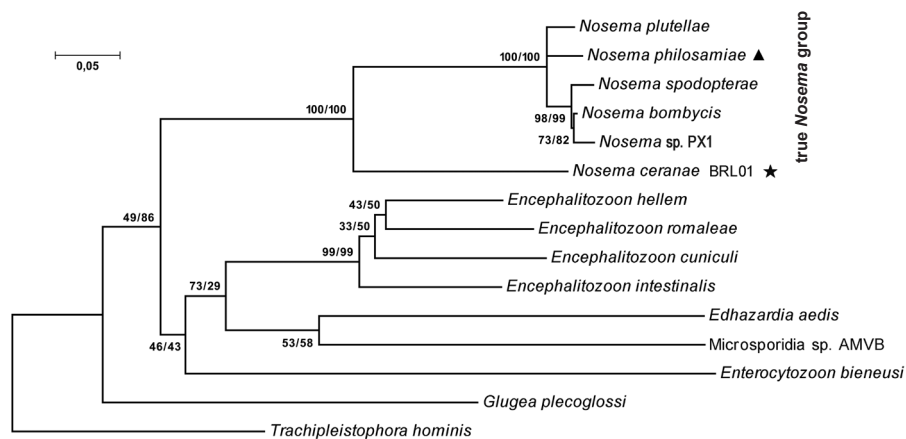
The partial gene of *N. philosamiae* beta-tubulin contained an open reading frame of 1 161 bp, with a GC content of 36.1%. The percent identity of pairwise comparisons based on beta-tubulin nucleotide sequences and amino acid sequences also revealed a high similarity (nucleotide sequences  $\geq$  96.0%; amino acid sequences  $\geq$  99.0%) among *N. philosamiae*, *N. bombycis*, *N. spodopterae*, *Nosema* sp. PX1 and *N. plutellae* (Tables S4, S5). However, the nucleotide sequences showed 80.3% identity with *N. ceranae* BRL01 and the amino acid sequences showed 91.1% identity with *N. ceranae* BRL01.

### Phylogenetic trees constructed from tubulin gene sequences

The ML and MP phylogenies inferred from the partial alpha-tubulin sequence alignment are based on the nucleotide alignment of 14 microsporidian sequences (Fig. 1). *N. philosamiae*, along with *N. bombycis*, *N. spodopterae*, *N. plutellae* and *Nosema* sp. PX1, forms a separate clade with a strong bootstrap value ( $> 99\%$ ), with *N. ceranae* BRL01 forming a clade of its own. This was further supported by genetic distances, i.e. the pairwise distance of alpha-tubulin nucleotide sequences between *N. philosamiae*, *N. bombycis*, *N. spodopterae*, *N. plutellae* and *Nosema* sp. PX1 varied from 0.013 to 0.037 and the pairwise distance of alpha-tubulin nucleotide sequences between *N. philosamiae* and *N. ceranae* BRL01 was found to be 0.264 (Table S2). The pairwise distance of alpha-tubulin amino acid sequences between *N. philosamiae*, *N. bombycis*, *N. spodopterae*, *N. plutellae*, and *Nosema* sp. PX1 varied from 0 to 0.005 and the pairwise distance of alpha-



**Fig. 1.** The maximum likelihood phylogenetic tree based on alpha-tubulin gene sequences. The bootstrap values of MP/ML for 1000 replicates are indicated at nodes. *Trachipleistophora hominis* was used as outgroup. *Nosema philosamiae* is indicated by triangle (▲) and *N. ceranae* BRL01 by pentagrams (★).



**Fig. 2.** The maximum likelihood phylogenetic tree based on beta-tubulin gene sequences. The bootstrap values of MP/ML for 1000 replicates are indicated at nodes. *Trachipleistophora hominis* was used as outgroup. *Nosema philosamiae* is indicated by triangle (▲) and *N. ceranae* BRL01 by pentagrams (★).

tubulin amino acid sequences between *N. philosamiae* and *N. ceranae* BRL01 was found to be 0.116 (Table S3).

The ML and MP phylogenies inferred from the partial beta-tubulin sequence alignment, based on 15 taxa, are shown in Figure 2. The beta-tubulin phylogeny shares many branches in common with that of alpha-tubulin. *N. philosamiae* and four species of the ‘true’ *Nosema* clustered reproducibly with a high bootstrap support of 100%. *Nosema ceranae* BRL01 formed a clade of its own with high bootstrap support (100%). The ML and MP phylogenies inferred from the combined alpha- and beta-tubulin sequence alignment include 13 taxa (Fig. 3). The combined analysis also indicated a similar relationship between the ‘true’ *Nosema* species and *N. ceranae* BRL01.

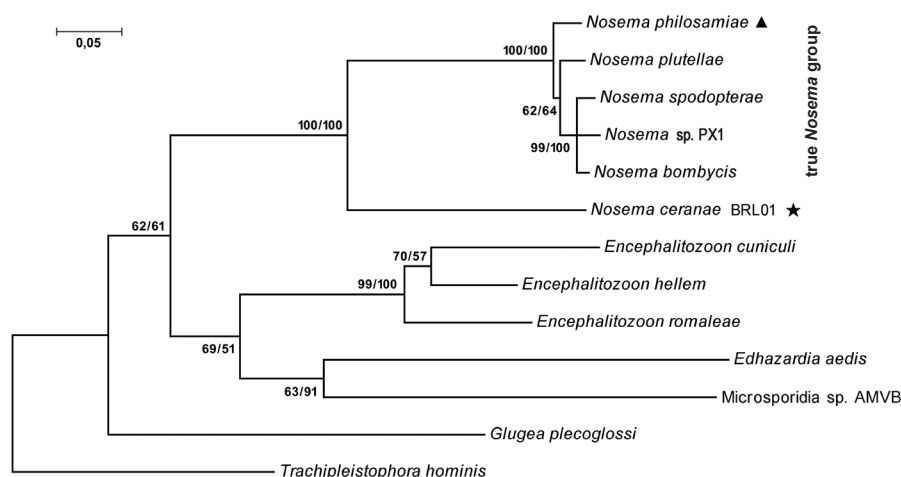
## DISCUSSION

The differential diagnosis of *Nosema* species was based on subtle differences in overlapping characteristics such as spore size, number of nuclei per cell, type of cell

division, microsporidium-host relationships, and primary site of infection. However, closely related species are often difficult to differentiate using morphological criteria alone (Wittner and Weiss 1999).

Recently, phylogenetic analysis based on the rRNA sequences suggested that the species that should be placed in *Nosema* (= ‘true’ *Nosema* species) should be separated from the *Nosema/Vairimorpha* group (Baker et al. 1994, Tsai et al. 2003, 2005, Kyei-Poku et al. 2008). The organization of the rRNA gene of ‘true’ *Nosema* species is 5'-LSU-ITS-SSU-3', such as that in *N. bombycis*, *N. spodopterae*, *N. plutellae*, *N. antheraeae* and *N. philosamiae* – Huang et al. (2004) Tsai et al. (2005), Wang et al. (2006), Ku et al. (2007), Zhu et al. (2010).

In contrast, *Nosema* species in the *Nosema/Vairimorpha* group, which includes *N. apis* Zander, 1909, *N. ceranae* and *N. thomsoni* Wilson et Burke, 1971, have the typical rRNA gene subunit arrangement (5'-SSU-ITS-LSU-3') (Gatehouse and Malone 1998, Huang et al. 2008, Kyei-Poku et al. 2008).



**Fig. 3.** The maximum likelihood phylogenetic tree based on combined alpha- and beta-tubulin gene sequences. The bootstrap values of MP/ML for 1000 replicates are indicated at nodes. *Trachipleistophora hominis* was used as outgroup. *Nosema philosamiae* is indicated by triangle (▲) and *N. ceranae* BRL01 by pentagrams (★).

In addition, the ‘true’ *Nosema* species, which infect only butterflies (Lepidoptera) are more closely related to each other than they are to other *Nosema* species (*Nosema* species in the *Nosema/Vairimorpha* group) that infect non-lepidopteran hosts (Baker et al. 1994, Zhu et al. 2011). Nageswara 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.

In the present study, phylogenies based on alpha-, beta- and combined alpha- plus beta-tubulin nucleotide data were in agreement with the relationships that have been proposed on the basis of analyses of rRNA sequences (Zhu et al. 2010). The sequence identity of alpha- and beta-tubulin genes indicated that *N. philosamiae* shares high sequence similarity with the other true *Nosema* species (nucleotide sequences  $\geq 96.0\%$ ; amino acid sequences  $\geq 99.0\%$ ), except for *N. ceranae* BRL01.

We found that the tubulin gene/protein sequences of *N. ceranae* BRL01 share low similarity (alpha-tubulin gene – 77.4–78.0% identity of the nucleotide sequences and 89.2% identity of the amino acid sequences; beta-tubulin gene – 79.9–81.1% identity of the nucleotide sequences and 90.6–91.1% identity of the amino acid sequences) with those of five other *Nosema* species, which share high se-

quence similarity (alpha-tubulin gene, 96.4–98.7% identity of the nucleotide sequences and 99.5–100% identity of the amino acid sequences, beta-tubulin gene, 96.0–98.7% identity of the nucleotide sequences and 98.2–100% identity of the amino acid sequences) with each other (Tables S2–S5).

The sequence analysis of alpha- and beta-tubulin gene appears to provide substantive evidence for the argument that *Nosema* species should be properly separated into two groups (‘true’ *Nosema*-species group and *Nosema/Vairimorpha* group) (Baker et al. 1994, Tsai et al. 2003, 2005, Dong et al. 2010, Kyei-Poku et al. 2011, Zhu et al. 2011).

In conclusion, the sequence identity and phylogenetic analysis based on alpha-, beta- and combined alpha- plus beta-tubulin gene sequences have indicated that *N. philosamiae* is a member of the ‘true’ *Nosema* group. Moreover, the alpha- and beta-tubulin sequences may be useful as a diagnostic tool to discriminate the ‘true’ *Nosema* group from the *Nosema/Vairimorpha* group.

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