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Phylogenetic Analysis of *Nosema antheraeae* (Microsporidia) Isolated from Chinese Oak Silkworm, *Antheraea pernyi*

LIN-LING WANG,[a] KE-PING CHEN,[a] ZE ZHANG,[b] QIN YAO,[a] GUI-TIAN GAO[a] and YUAN ZHAO[a]

[a]*Institute of Life Sciences, The University of JiangSu, ZhenJiang 212013, China, and*
[b]*The Key Sericultural Laboratory of Agricultural Ministry, Southwest University, Chongqing 400716, China*

ABSTRACT. The microsporidian *Nosema antheraeae* is a pathogen that infects the Chinese oak silkworm, *Antheraea pernyi*. We sequenced the complete small subunit (SSU) rRNA gene and the internal transcribed spacer (ITS) of *N. antheraeae*, and compared the SSU rRNA sequences in other microsporidia. The results indicated that *Nosema* species, including *N. antheraeae*, formed two distinct clades, consistent with previous observations. Furthermore, *N. antheraeae* is clustered with *N. bombycis* with high bootstrap support. The organization of the rRNA gene of *N. antheraeae* is LSU–ITS1–SSU–ITS2-5S, also following a pattern similar to the *Nosema* type species, *N. bombycis*. Thus, *N. antheraeae* is a *Nosema* species and has a close relationship to *N. bombycis*.

Key Words. ITS, *Nosema bombycis*, phylogenetic analysis, small subunit rRNA.

OVER the past decade there has been increasing interest in studying the phylogeny of microsporidia at the molecular level because, as parasites, they have a broad range of hosts from invertebrates to vertebrates, including insects, fishes, and mammals (Baker et al. 1995; Hatakeyama et al. 1997, 2000; Keeling, Luker, and Palmer 2000; Rao, Nath, and Saratchandra 2005; Rao et al. 2004; Tsai et al. 2003). Microsporidia cause considerable problems in industries such as fisheries and sericulture. Molecular evidence has demonstrated that microsporidia are phylogenetically related to the fungi (Fast, Logsdon, and Doolittle 1999; Hirt et al. 1999; Keeling and Doolittle 1996; Keeling et al. 2000; Vossbrinck et al. 1987).

The microsporidian *Nosema antheraeae* is a pathogen that infects the Chinese oak silkworm, *Antheraea pernyi*, causing pebrine disease (Ding et al. 1998). This disease is the key factor obstructing the developmental progress of sericulture in China. Much work has concentrated on the morphology, pathology, and biology of *N. antheraeae*. However, molecular biological work is limited. Therefore, we sequenced the small subunit (SSU) rRNA gene and the internal transcribed spacer (ITS) of *N. antheraeae*. By comparing the SSU rRNA of *N. antheraeae* with the corresponding sequences of 35 other microsporidia, we present here the molecular biological evidence of the taxonomy of *N. antheraeae*.

MATERIALS AND METHODS

Spore purification and sequencing. Infected Chinese oak silkworms were collected from YunYang Sericultural Experimental Station, HeNan, China. The protocols for spore purification and DNA extraction follow previous descriptions (Huang et al. 1998; Tsai et al. 2002). The PCR reactions and sequencing were carried out as described previously (Huang et al. 2004).

Phylogenetic analysis. Thirty-six SSU rRNA nucleotide sequences were aligned using CLUSTALX version 1.8 (Thompson et al. 1997). PAUP* version 4b.10 (Swofford 2003) was used to search for the most-parsimonious tree. Maximum likelihood (ML) analysis was performed by quartet puzzling using TREE-PUZZLE 5.0 (Schmidt et al. 2002) under the default options. Neighbor-joining tree was reconstructed using MEGA 3.0 (Kumar, Tamura, and Nei 2004) under the two-parameter distance of Kimura (1980).

Corresponding Author: Ke-Ping Chen, Institute of Life Sciences, The University of JiangSu, ZhenJiang 212013, China—Telephone number: +86-0511-8791923; FAX number: +86-0511-8791923; e-mail: kpchen@ujs.edu.cn

RESULTS AND DISCUSSION

The sequence and organization of *Nosema antheraeae* rRNA. The DNA sequence of *N. antheraeae* rRNA obtained in the present study contains 2,108 bp and was submitted to GenBank (Accession number: DQ073396). Starting from the 5′-end, it includes part of the large subunit gene (LSU rRNA; 1–287 bp), the ITS (ITS1; 288–479 bp), the SSU gene (SSU rRNA: 480–1,716 bp), the ITS (ITS2; 1,717–1,993 bp), and the 5S rRNA gene (1,994–2,108 bp).

The *N. antheraeae* SSU rRNA contains 1,235 bp and has 34.33% GC. It is 99.1% similarity to the *Nosema* type species, *N. bombycis* (AY259631). The alignment of the two sequences suggests that there are two indels, five transitions, and three transversions.

The ITS region of *N. antheraeae* includes an ITS1 of 192 bp and an ITS2 of 278 bp, having 16.67% and 21.94% GC, respectively. The ITS1 is 57.8% similarity to ITS2.

The organization of the rRNA genes of *N. antheraeae* is LSU–ITS1–SSU–ITS2–5S, a pattern similar to those of *N. bombycis* (Huang et al. 2004) and of *N. spodopterae* (Tsai, Huang, and Wang 2005). The organization differs from that of other *Nosema* species and other genera of microsporidia. For example, the organization of the rRNA gene of *Nosema* species (AY383655) is SSU–ITS–LSU. However, the organization of most microsporidial rRNA genes is also SSU–ITS–LSU, e.g. *Encephalitozoon cuniculi* (Peyretaillade et al. 1998), *Encephalitozoon hellem* (Franzen et al. 1998), *Encephalitozoon intestinalis* (Zhu et al. 1994), and *Pleistophora ehrenbaumi* (Nilsen, Endresen, and Hordvik 1998), *Pleistophora typicalis* (Nilsen et al. 1998), *Pleistophora mulleri* (Terry et al. 2003), *Pleistophora* sp. (Nilsen et al. 1998). As the organization of these three *Nosema* species differs from that of most prokaryotes as well as eukaryotes, it is quite unusual.

Phylogenetic analysis by using the SSU rRNA gene sequences. Since a previous phylogenetic analysis used *Amblyospora connecticus* and *Culicosporella lunata* as the outgroup taxa for the microsporidia (Rao et al. 2004), the same taxa were used as outgroups in the present study. The alignment of 36 sequences was 1,169 bp in length with some indels. The data matrix for the 36 taxa, including the two outgroup taxa, contained 747 variable sites, of which 642 were phylogenetically informative. Parsimony analysis produced 20,894 maximum parsimony (MP) trees of 2,262 steps. The strict consensus MP tree had a consistency index of 0.5752, a retention index of 0.8464.

We also used ML and neighbor-joining methods to reconstruct the phylogenetic trees for the same sequence data. The topologies of the resultant phylogenetic trees are essentially the same as that of the MP phylogenetic tree, and the topologies are quite robust as

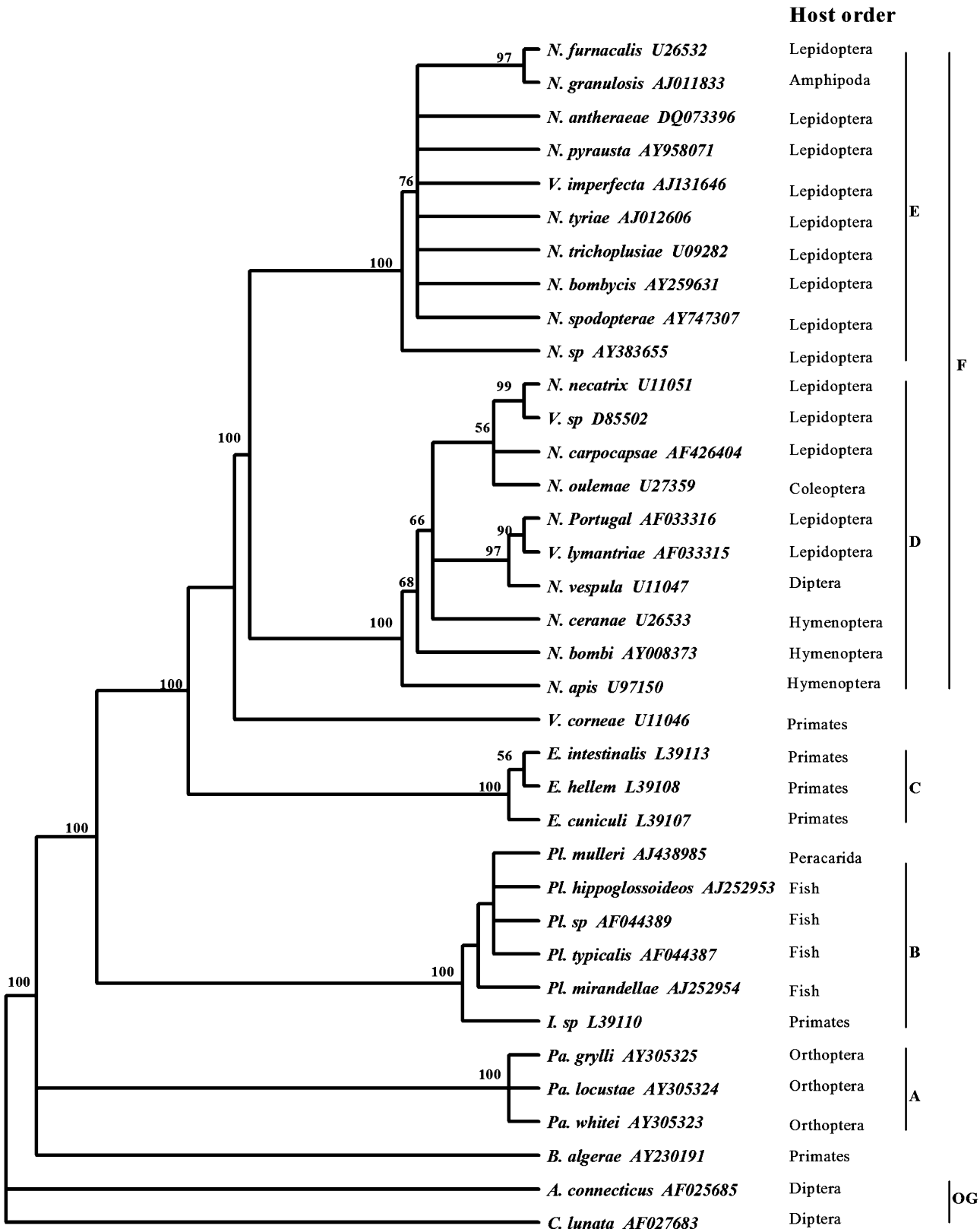

Fig. **1.** Strict consensus tree of the 20,894 most-parsimonious trees of 2,262 steps derived from an equally weighted maximum parsimony analysis of the small subunit rRNA nucleotide sequences of 34 ingroup taxa and two outgroup (OG) taxa (Consistency Index = 0.5752; Retention Index = 0.8464). Bootstrap percentages with 100 resamplings appear above the branches; the values $<50\%$ are not indicated. *N.*, *Nosema*; *V.*, *Vittaforma*; *Pl.*, *Pleistophora*; *Pa.*, *Paranosema*; *I.*, *Ichthyosporidium*; *E.*, *Encephalitozoon*; *B.*, *Brachiola*; *A.*, *Amblyspora*; *C.*, *Culicosporella*. GenBank Accession number for each sequence is shown behind the corresponding species name.

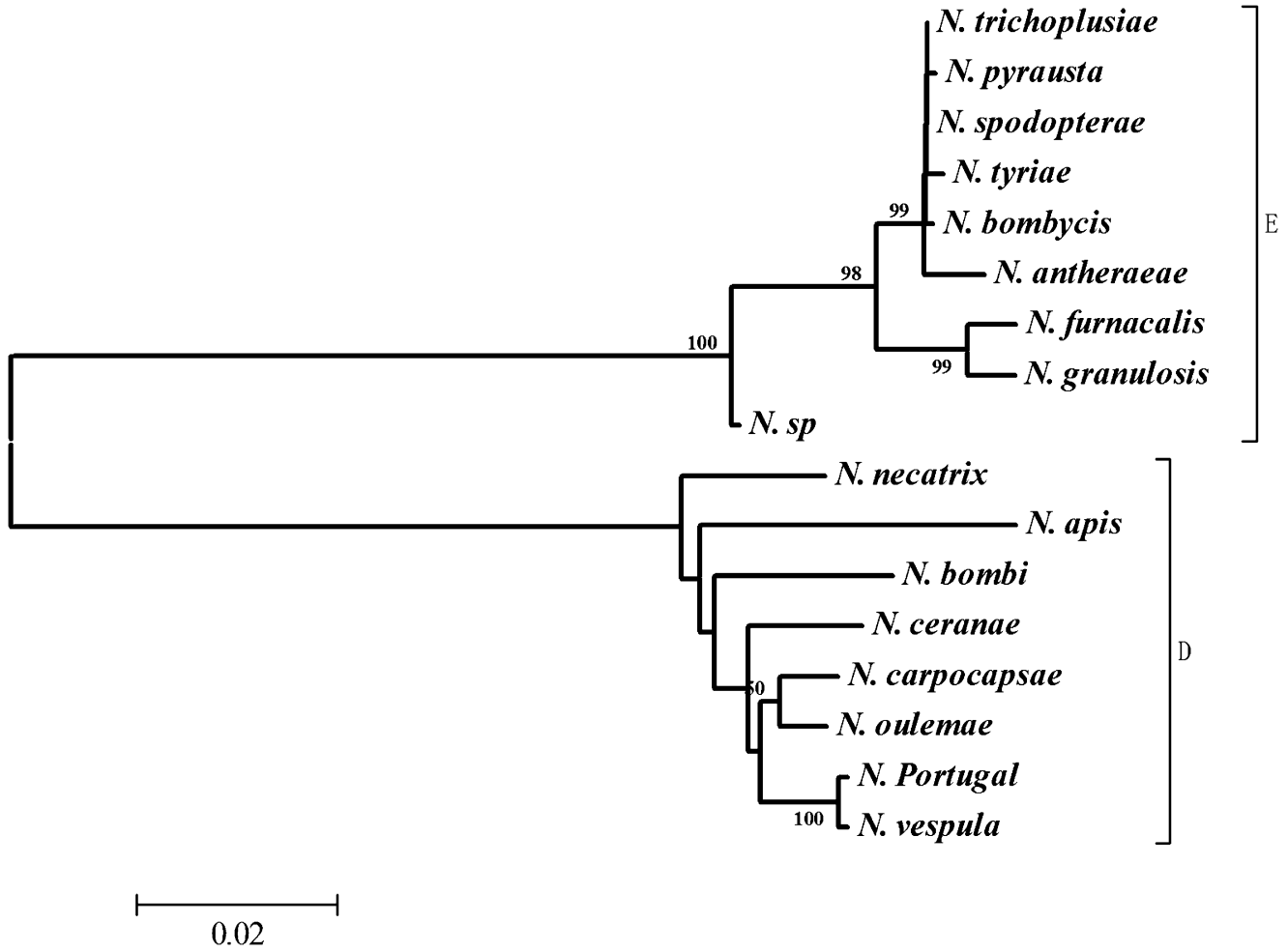

Fig. **2.** A neighbor-joining tree for 17 *Nosema* species. The tree was generated by the MEGA 3 software with Kimura 2-parameter distances and 1,000 bootstrap replicates. Bootstrap values $< 50\%$ are not indicated.

indicated by the bootstrap support (Fig. 1). *Nosema* forms two distinct clades D and E with high bootstrap support (Fig. 1). Clade E includes *N. bombycis* and was formed by *Nosema* species that parasitize lepidopterans, except for *N. granulosis*, which parasitizes Amphipoda. Clade D includes *Nosema* species that parasitize Lepidoptera, Diptera, Coleoptera, and Hymenoptera (see also Rao et al. 2004). In addition, microsporidia that parasitize primates, fish, and Orthoptera also form three tight clusters (A, B, C in Fig. 1), all with 100% bootstrap value.

To examine more closely the relationships among *Nosema* species, we constructed a phylogenetic tree using the SSU rRNA sequences from 17 *Nosema* (i.e. cluster F in Fig. 1). The branching pattern is basically the same as that of the Clade F (cf. Fig. 1, 2). However, *N. antheraeae*, *N. bombycis*, *N. tyriae*, *N. spodoptera*, *N. pyrausta*, and *N. trichoplusiae* together formed a cluster within Clade E with 99% bootstrap support, indicating that *N. antheraeae* is closely related to *N. bombycis*.

We observed huge variation in the lengths of available ITS sequences for the microsporidia. The sizes of the ITS region between the LSU rRNA and SSU rRNA range from 27 to 36 bp among *Encephalitozoon* species, from 32 to 45 bp among *Pleistophora* species, and from 33 to 192 bp among *Nosema* species. The sizes may be more variable among *Nosema* species due to different organization of the rRNA genes. Because the lengths of the ITS of most microsporidia range from 27 to 45 bp, the alignment of ITS sequences is too short to be phylogenetically useful. However, *N. antheraeae*, *N. bombycis*, and *N. spodoptera* have ITS regions of similar lengths with 192, 179, and 185 bp, respectively, greatly differing from other microsporidia. This, together with their placement in the same SSU rRNA clade (Fig. 2), suggests that these three species are closely related to each other.

Nosema antheraeae has a life cycle substantially similar to the type species, *N. bombycis* (Ding et al. 1998; Su and Ding 2003; Zhang et al. 1996). Therefore, based on the evidence from the life cycle, our phylogenetic analyses, and the organization of the rRNA gene, we can conclude that *N. antheraeae* is a *Nosema* species, closely related to *N. bombycis*.

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LITERATURE CITED

Baker, M. D., Vossbrinck, C. R., Didier, E. S., Maddox, J. V. & Shadduck, J. A. 1995. Small subunit ribosomal DNA phylogeny of various microsporidia with emphasis on AIDS related forms. *J. Eukaryot. Microbiol.*, **42**:564–570.

Ding, J., Su, G., Zhang, Y. & Yu, G. 1998. Study on infectivity of larva of *Nosema antheraeae* to histiocyte of Chinese oak silkworm larvae and multiplication. *LiaoNing Agric. Sci.*, **4**:6–10.

Fast, N. M., Logsdon, J. M. & Doolittle, W. F. 1999. Phylogenetic analysis of the TATA box binding protein (TBP) gene from *Nosema tyriae*: evidence for a microsporidia–fungi relationship and spliceosomal intron loss. *Mol. Biol. Evol.*, **16**:1415–1419.

Franzen, C., Muller, P., Hartmann, P., Hegener, M. & Schrappe, V. 1998. Polymerase chain reaction for diagnosis and species differentiation of microsporidia. *Folia Parasitol. (Prague)*, **45**:140–148.

Hatakeyama, Y., Bansal, A. K., Iwano, H., Kawakami, Y. & Ishihara, R. 2000. Characterisation of SSU-rRNA sequence of a new microsporidium, *Nosema* sp. (Nosematidae: Microsporidia), isolated from *Antheraea mylitta* Drury (Lepidoptera: Saturniidae) in India. *Indian J. Seric.*, **39**:131–134.

Hatakeyama, Y., Kawakami, Y., Iwano, H., Inoue, T. & Ishihara, R. 1997. Analysis and taxonomic inference of small subunit ribosomal RNA sequences of five microsporidia pathogenic to the silkworm, *Bombyx mori*. *J. Seric. Sci. Jpn.*, **66**:242–252.

Hirt, R. P., Logsdon, J. M., Healy, B., Dorey, M. W., Doolittle, W. F. & Embley, T. M. 1999. Microsporidia are related to fungi: evidence from the largest subunit of RNA polymerase II and other proteins. *Proc. Natl. Acad. Sci. USA*, **96**:580–585.

Huang, H. W., Lo, C. F., Tseng, C. C., Peng, S. E., Chou, C. M. & Kou, C. H. 1998. The small subunit ribosomal RNA gene sequence of *Pleistophora anguillarum* and the use of PCR primers of diagnostic detection of the parasite. *J. Eukaryot. Microbiol.*, **4**:556–560.

Huang, W. F., Tsai, S. J., Lo, C. F., Soichi, Y. & Wang, C. H. 2004. The novel organization and complete sequence of the ribosomal RNA gene of *Nosema bombycis*. *Fungal Genet. Biol.*, **41**:473–481.

Keeling, P. J. & Doolittle, W. F. 1996. Alpha-tubulin from early-diverging eukaryotic lineages and the evolution of the tubulin family. *Mol. Biol. Evol.*, **13**:1297–1305.

Keeling, P. J., Luker, M. A. & Palmer, J. D. 2000. Evidence from beta-tubulin phylogeny that microsporidia evolved from within the fungi. *Mol. Biol. Evol.*, **17**:23–31.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, **16**:111–120.

Kumar, S., Tamura, K. & Nei, M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.*, **5**:150–163.

Nilsen, F., Endresen, C. & Hordvik, I. 1998. Molecular phylogeny of microsporidians with particular reference to species that infect the muscle of fish. *J. Eukaryot. Microbiol.*, **45**:535–543.

Peyretaillade, E., Biderre, P., Peyret, F., Duffieux, G. & Metenier, M. 1998. Microsporidian *Encephalitozoon cuniculi*, a unicellular eukaryote, with an unusual chromosomal dispersion of ribosomal genes and a LSU rRNA reduced to the universal core. *Nucleic Acids Res.*, **26**: 3513–3520.

Rao, S. N., Muthulakshmi, M., Kanginakudru, S. & Nagaraju, J. 2004. Phylogenetic relationships of three new microsporidian isolates from the silkworm, *Bombyx mori*. *J. Invertebr. Pathol.*, **86**:87–95.

Rao, S. N., Nath, B. S. & Saratchandra, B. 2005. Characterization and phylogenetic relationships among microsporidia infecting silkworm, *Bombyx mori*, using inter simple sequence repeat (ISSR) and small subunit rRNA (SSU-rRNA) sequence analysis. *Genome*, **48**:355–366.

Schmidt, H. A., Strimmer, K., Vingron, M. & von Haseler, A. 2002. TREE-PUZZLE: Maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics*, **18**:502–504.

Su, G. & Ding, J. 2003. Study on infectivity of *Nosema antheraeae* to histiocyte of Chinese oak silkworm larvae and multiplication. *North. Agric. Sci.*, **24**:34–35.

Swofford, D. L. 2003. PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4.0b10. Sinauer Associates, Sunderland, MA.

Terry, R. S., MacNeil, C., Dick, J. T. A., Smith, J. E. & Dunn, A. M. 2003. Resolution of a taxonomic conundrum: an ultrastructural and molecular description of the life cycle of *Pleistophora mulleri* (Pfeiffer 1895; Georgevitch 1929). *J. Eukaryot. Microbiol.*, **50**:266–273.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997. The CLUSTAL-X windows interference: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, **25**:4876–4882.

Tsai, S. J., Huang, W. F. & Wang, C. H. 2005. Complete sequence and gene organization of the *Nosema spodopterae* rRNA gene. *J. Eukaryot. Microbiol.*, **52**:52–54.

Tsai, S. J., Kou, G. H., Lo, C. F. & Wang, C. H. 2002. Complete sequence and structure of ribosomal RNA gene of *Heterosporis anguillarum*. *Dis. Aquat. Org.*, **49**:199–206.

Tsai, S. J., Lo, C. F., Soichi, Y. & Wang, C. H. 2003. The characterization of Microsporidian isolates (Nosematidae: Nosema) from five important lepidopteran pests in Taiwan. *J. Invertebr. Pathol*, **83**:51–59.

Vossbrinck, C. R., Baker, M. D., Didier, E. S., Debruuner-Vossbrinck, B. A. & Woese, C. R. 1987. Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. *Nature*, **326**:411–414.

Zhang, Y., Ding, J., Yu, G. & Su, G. 1996. The relationship of pebrine of *Antheraca perny* and field insects. *LiaoNing Agric. Sci.*, **5**:31–34.

Zhu, X., Wittner, M., Tanowitz, H. B., Cali, A. & Weiss, L. M. 1994. Ribosomal RNA sequences of *Enterocytozoon bieneusi*, *Septata intestinalis* and *Ameson michaelis*: phylogenetic construction and structural correspondence. *J. Eukaryot. Microbiol.*, **41**:204–209.

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