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Full Length Research Paper

Improving phylogenetic inference of microsporidian *Nosema antheraeae* among *Nosema* species with RPB1, α-and β-tubulin sequences

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The microsporidian *Nosema antheraeae* is an intracellular parasite that infects the Chinese oak silkmoth, *Antheraea pernyi*. However, its phylogenetic position among the *Nosema* isolates is not yet clear. For a better understanding of the phylogenetic relationships between *Nosema* species, the RPB1, α-tubulin and β-tubulin genes of *N. antheraeae* were sequenced and analyzed. These data showed that the *N. antheraeae* clade was a sister to the clade of *Nosema bombycis* and other lepidopteran-infecting *Nosema* spp. Moreover, even if their respective silkmoth hosts, *A. pernyi* and *Bombyx mori*, were known to be close relatives (both belong to superfamily, *Bombycoidea*), *N. antheraeae* was not more closely related to *N. bombycis* than to other lepidopteran *Nosema* species. Therefore, the relationship between silkmoth-infecting *Nosema* species was not in accordance with the phylogeny of the silkmoth hosts; this might indicate that cospeciation have few influence on the silkmoth-infecting *Nosema* spp.

Key words: *Nosema antheraeae*, *Nosema bombycis*, RPB1, tubulin, phylogenetic.

INTRODUCTION

Chinese oak silkworm (Lepidoptera: *Bombycoidea*: *Antheraea pernyi*) is an economically important insect mainly cultivated in Southeast Asia and it can be widely utilized for silk production and food. The microsporidian *Nosema antheraeae* is an intracellular parasite that infects the Chinese oak silkmoth, and many studies have focused on the morphology and pathology of *N. antheraeae* (Ding et al., 1998; Li et al., 2005). How-ever, molecular analysis of the relationship between *N. antheraeae* and other microsporidia have been, so far, based solely on the small subunit of rRNA, which indicated that *N. antheraeae* was a *Nosema* type species (Wang et al., 2006) and its phylogenetic position among other species of *Nosema* were still poorly understood. Up till now, the phylogenetic relationships of *Nosema* speices were seldom clearly described in previous studies. The consensus phylogenetic trees based on the 18S rDNA and RPB1 (largest subunit of RNA polymerase II) sequences show that most *Nosema* species can be divided into two well-supported clades (Ironside, 2007). Another phylogenetic analysis also shows that most lepidopteran-infecting *Nosema* species can be clustered into one group (Ku et al., 2007). However, no further information about the phylogenetic affinities of most *Nosema* species was provided in these studies. Recently, a cophylogenetic relationship between three bee-infecting *Nosema* species (*Nosema ceranae*, *Nosema bombi* and *Nosema apis*) and bees was analyzed, suggesting that both cospeciation and a host-switch had occurred during the evolution of these three *Nosem*a species (Shafer et al., 2009). The α-tubulin, β-tubulin and RPB1 genes were usually used to determine the phylogenetic relationships among microsporidia in previous studies (Edlind et al., 1996; Keeling, 2003; Ku et al., 2007; Lee et al., 2008), so, we sequenced these three genes of *N. antheraeae* to clearly elucidate the relationship between *N. antheraeae* and the other *Nosema* species. Moreover, we provided

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Abbreviations: CTAB, Cetyl trimethylammonium bromide; **PCR,** polymerase chain reaction; **ML,** maximum likelihood; **NJ,** neighbor joining; **GTR,** general time reversible; **IGS,** intergenic spacer.

the new opinion about the affinity between *N. antheraeae* and *Nosema bombycis,* both of which are parasites of the two economically important silkmoths, *A. pernyi* and *Bombyx mori*, respectively.

MATERIALS AND METHODS

Isolate collection and spore purification

The pupae of *A. pernyi* with pebrine disease were collected from a farm in Yunyang Town in Henan Province, China (longitude: 112.53; longitude: 33.01). The silk gland was dissected, homogenized and centrifuged for spore enrichment. The spores were purified using a discontinuous Percoll gradient (25, 50, 75 and 100%, v/v) and centrifuged at 30,000 × *g* for 40 min. Spore bands in the 75% gradient layer were harvested and stored at 4°C.

DNA preparation, polymerase chain reaction (PCR) and sequencing

The genomic DNAs of 10^9 *N. antheraeae* spores were extracted with cetyl trimethylammonium bromide (CTAB) method (Li and Wang, 2006). The RPB1, α-tubulin and β-tubulin genes of *N. antheraeae* were amplified using the primer sets 5′-RRHGG RGARMTHDTRTCDGGW-3′ and 5′-GTCATRTCHGTHGCWGGTT CR -3′ for the RPB1 gene, 5′-YTRTACWSHAARGARCA YRRN-3′ and 5′-CCRTCN CCHACYTACCAYTGB-3′ for the α-tubulin gene, and 5′-RTVGGWNSMAARTTYT GGGAR-3′ and 5′-VCCRTCD RSDGTYTACCAYTG-3′ for the β-tubulin gene. These primers were designed using gene sequences of *N. bombycis* and other lepidopteran- infecting *Nosema* species obtained from the publicly available Genbank database. PCR amplifications were performed using an Eppendorf Mastercycle apparatus. Each 50 µl reaction contained 1 µl genomic DNA (50 ng/µl), 2 µl of each primer (10 µmol/l), 2 µl dNTPs (10 mmol/l), 2 µl $MgCl_2$ (25 mmol/l), 5 µl 10 × PCR buffer and 1 µl Taq DNA polymerase (5 U/µl, TaKaRa Co, Japan).

Samples were heated to 94°C for 5 min to denature the DNA, then 30 cycles were run with 1 min of denaturation at 94°C, 30 s of annealing at 57°C(56°C for β-tubulin gene; 61°C for RPB1 gene) and 2 min of extension at 72°C. The final extension step was carried out at 72°C for 10 min. PCR products that were well separated on agarose gels were excised and purified using the gel extraction kit (Omega Bio-Tek, USA) following the manufacturer's instructions. The amplicons were then cloned into a pMD19-T vector with the TA Cloning Kit (TaKaRa Biotechnology, Dalian, China). Two independent clones were sequenced using Big Dye chemistry with ABI3730 (Applied Biosystems, Foster CA, USA). The *N. antheraeae* RPB1, α-tubulin and β-tubulin gene sequences were deposited into the Genbank database with accession numbers HQ215548, HQ215549 and HQ215550, respectively.

Phylogenetic and molecular evolution analysis

The RPB1, α-tubulin and β-tubulin gene sequences of *N. antheraeae* and all the other *Nosema* species available on the Genbank database were aligned using Clustal X and also checked manually. The RPB1 alignment was obtained for 13 taxa containing 1475 characters; the α-tubulin alignment, for 10 taxa containing 1160 characters; and the β-tubulin alignment, for 12 taxa containing 1171 characters. Phylogenetic trees were then constructed using the methods of Phyml 2.4.4 maximum likelihood (ML) (Guindon and Gascuel, 2003), MEGA 4.0 Neighbor joining (NJ) (Tamura et al., 2007) and MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001). The yeast *Schizosaccharomyces pombe* was chosen as the outgroup for three rooted trees with 100 bootstrap replicates.

The best-fit model of DNA substitution for each phylogenetic tree was selected using the MultiPhyl v. 1.0 (Keane et al., 2007). Analyses for ML and NJ were performed using the general time reversible (GTR) (Yang, 1994) and maximum composite likelihood substitution models (Tamura et al., 2007), respectively. Meanwhile, for both ML and NJ analyses, the number of the gamma rate categories was set at eight, and the gamma distribution parameter was determined to be 2.0. For the Bayesian analysis, GTR substitution models were chosen, and then the MrBayes program was run for 1×10^7 generations and sampled every 100 generations. The burn-in period discarded 2,500,000 generations. Posterior clade probabilities were used to assess nodal support.

Nucleotide differences of rDNA between species were represented by the percentage identity of the entire SSUrRNA, IGS, ITS and 5S. Sequence identities and the number of base changes were calculated by bl2seq program (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Phylogenetic position of *N. antheraeae*

Phylogenies of RPB1, α-tubulin and β-tubulin gene sequences created using the three inferred methods showed very similar phylogenetic relationships among the *Nosema* spp. Some relationships were recovered as expected; that is, lepidopteran-infecting species were found to be most closely related and tended to form a clade. The phylogenetic trees generated using the α-tubulin and β-tubulin gene sequences showed that *N. bombycis*, *Nosema spodopterae,* and *Nosema* sp. PX1 were grouped together in a single clade which was a sister to the *N. antheraeae* clade (Figure 1). The phylogenetic trees generated using RPB1 gene sequences also showed that *N. antheraeae* was a unique sister group to the clade comprised of *N. bombycis* and other lepidopteran-infecting *Nosema* species (Figure 2). It is notable that *N. bombycis* were found to be more distantly related to *N. antheraeae* than *Nosema* PX1, even if both *N. bombycis* and *N. antheraeae* were silkmoth-infecting *Nosema* species. The host of *N. bombycis* was domesticated silkworm *(B. mori)*, and the host of *N.antheraeae* was wild silkworm*, A. pernyi.* It had been proven that silkmoth hosts *A. pernyi* and *B. mori* were most closely related than other non-silkworm hosts (Regier et al., 2005; Arunkumar et al., 2006). Therefore, all our phylogenetic results indicated that even if their respective hosts had very close evolutionary origins, *N. antheraeae* was not more closely related to *N. bombycis* than to other lepidopteran-infecting *Nosema* species, proving that the relationship between silkmoth-infecting *Nosema* species was not in accordance with the phylogeny of the silkmoth hosts.

Sequence variation

To further evaluate the affiliation between *N. antheraeae*,

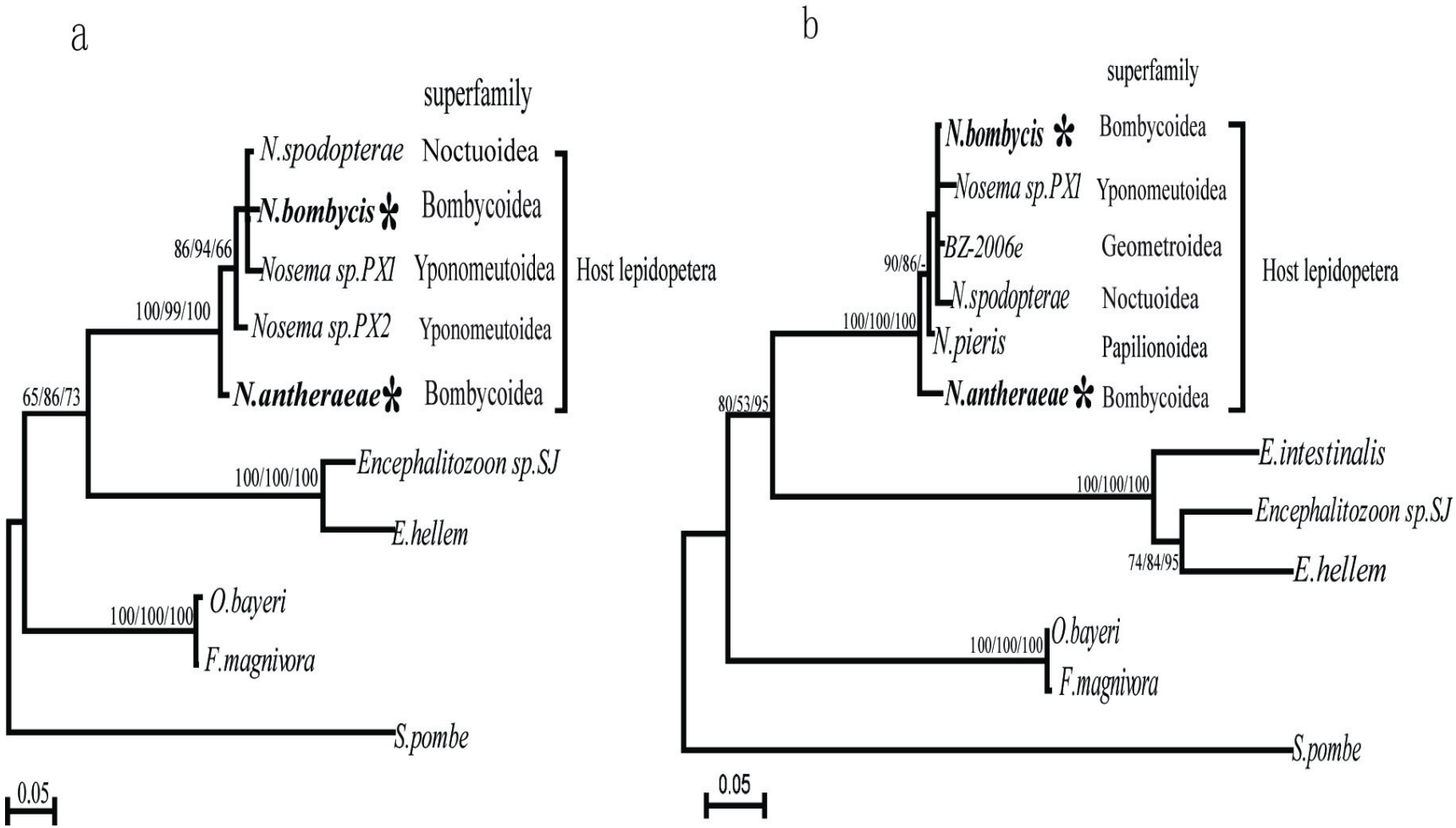

Figure 1. Phylogenetic analysis of the microsporidian α-tubulin and β-tubulin sequences. (a) The ML, NJ and Bayesian method for the α-tubulin tree; (b) The ML, NJ and Bayesian method for the β-tubulin tree. The bootstrap values for both trees were ML/NJ/Bayesian and values <50% are not shown. *N., Nosema; E., Encephalitozoon; O., Octosporea; F., Flabelliforma; S. lophii, Spraguea lophii; S. pomb, Schizosaccharomyces pomb*.

N. bombycis, *Nosema* sp. PX1, and *N. spodopterae*, we analyzed the nucleotide differences in their rDNA sequences. The results showed that the homologous rDNA intergenic spacer (IGS) region between *N. antheraeae* and *Nosema* sp. PX1 or between *N. antheraeae* and *N. spodopterae* was much more divergent than that between *N. bombycis* and *Nosema* sp. PX1 or *N. bombycis* and *N. spodopterae* (as seen in Table 1); this was consistent with the above results, which indicated that *N. antheraeae* was not more

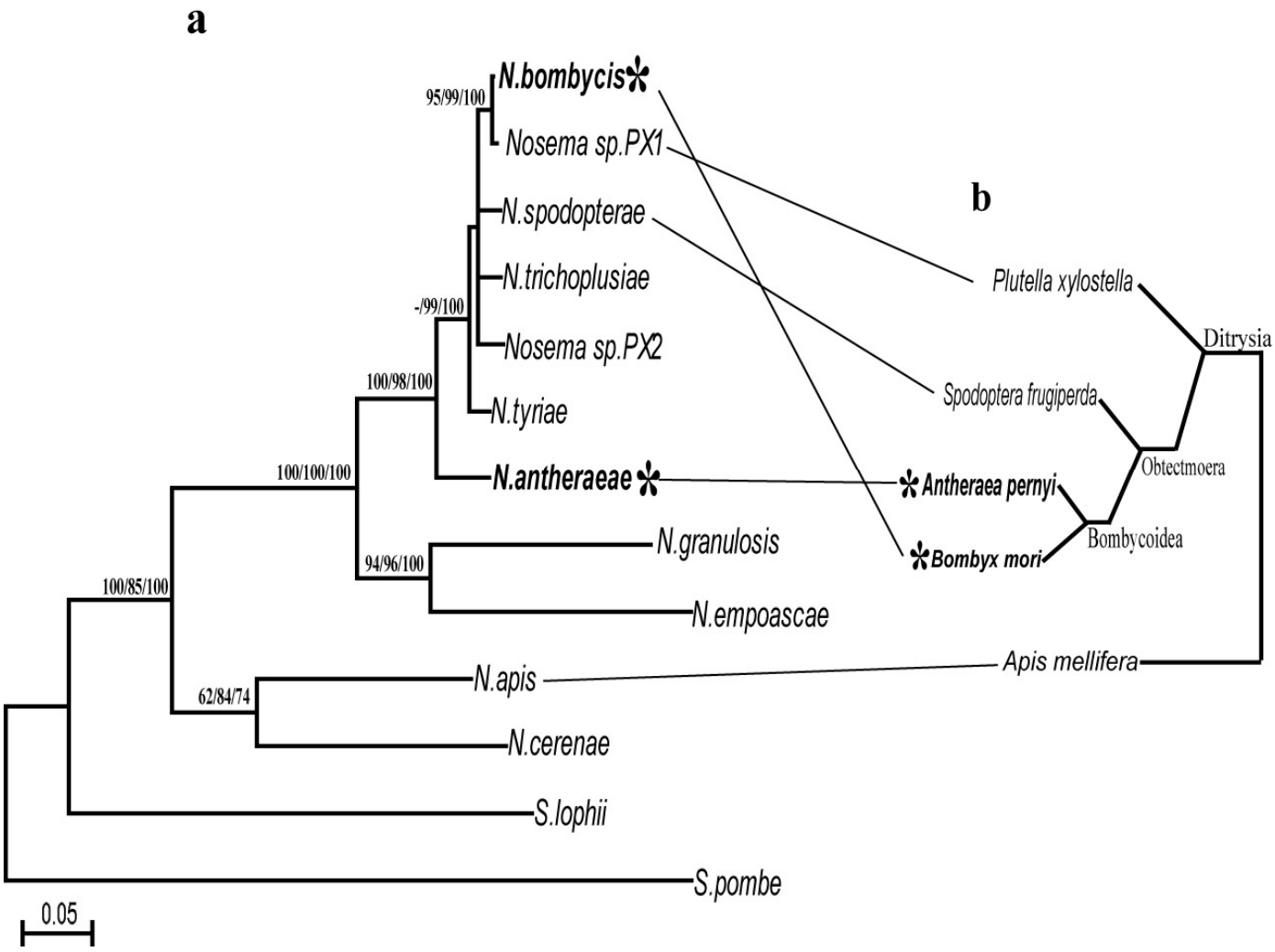

Figure 2. Phylogenetic analysis of Nosema RPB1 sequences illustrating host-parasite association. Associated taxa are joined by diagonal lines. (a) The ML, NJ and Bayesian method for RPB1 trees and values <50% are not shown; (b) Representation of close relationship of several insects host by referring to the previous study (Regier et al., 2005). The lengths of branch were not shown in scale and did not denote actual phylogenetic distance. The taxonomic names were shown under each node of the clade.

closely related to *N. bombycis* than to *Nosema* sp. PX1 or *N. spodopterae.*

DISCUSSION

N. bombycis was previously thought to be more closely related to *N. antheraeae* for the following reasons: First, their hosts, *A. antheraea and B. mori*, respectively, both belong to the *Bombycoidea* superfamily; second, the SSU rDNA sequence of *N. antheraeae* showed marked similarity (98%, 1331/1346) to that of *N. bombycis* (Wang et al., 2006); and third, the life cycle of *N. antheraeae* was very similar to that of *N. bombycis* (Zhang et al., 1996; Ding et al.,1998; Su and Ding, 2003). Here, our studies based on the tubulin and RPB1 genes sequences had improved phylogenetic inference of *N. antheraeae* and further showed that *N. bombycis* was not more closely related to *N. antheraeae* than to the other lepidopteran-infecting *Nosema* species. However, their respective host, *A. pernyi* and *B. mori* had a more recent common ancestor. This indicated that cospeciation have few influence on the silkmoth-infecting *Nosema* species. In our studies, *N. bombycis*, *Nosema* PX1 and *N. spodopterae* usually form a common clade, so, it might be hypothesized that the ancestor of *Nosema* PX1 or *N. spodopterae* had horizontally transmitted to *B. mori* and evolved into *N. bombycis*.

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Table 1. Sequence identities (%) of rRNA from *N. antheraeae*, *N. bombycis*, *N. spodopterae and Nosema* sp. PX1.

	Na			Nb			Ns			PX1		
	IGS	**SSU**	**ITS_5S**	**IGS**	**SSU**	**ITS_5S**	**IGS**	**SSU**	**ITS_5S**	**IGS**	**SSU**	**ITS_5S**
Na				79 (37)	99 (3)	96 (4)	75 (45)	99 (2)	93 (8)	77 (44)	99 (2)	94 (7)
Nb	79 (37)	99 (3)	96 (4)				89 (28)	99 (1)	96 (5)	92 (20)	99 (1)	96 (5)
Ns	75 (45)	99 (2)	93 (8)	89 (28)	99 (1)	96 (5)				92 (14)	100 (0)	95 (6)
PX1	77 (44)	99 (2)	94 (7)	92 (20)	99 (1)	96 (5)	92 (14)	100 (0)	95 (6)			

Na*, N. antheraeae*; Nb*, N. bombycis;* Ns, *N. spodopterae;* PX1*, Nosema. s*p. PX1. Sequence identities represent the percentage identity of the entire SSUrRNA, IGS, ITS and 5S. Numbers in parentheses show the number of base changes between species.

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