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Short Communication

Phylogenetic analysis of two putative *Nosema* isolates from Cruciferous Lepidopteran pests in Taiwan

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Abstract

In this study, a new microsporidian, PX2, was isolated from the diamondback moth, *Plutella xylostella*, and then compared with another isolate (PX1), and with *Nosema spodopterae* and *N. bombycis.* Sequence data showed that the rRNA gene organizations of PX1 and PX2 exhibited a typical *Nosema*-specific organization: 5′-LSUrRNA (large subunit ribosomal RNA)-ITS (internal transcribed spacer)-SSUrRNA-IGS (intergenic spacer)-5S-3′. Phylogenetic analysis (maximum likelihood, neighbor joining, maximum parsimony, and Bayesian analysis) of the LSUrRNA and SSUrRNA gene sequences, and the sequences of the alpha-tubulin, beta-tubulin, and RPB1 (DNA dependent RNA polymerase II largest subunit) genes found that PX1 was closer to *N. bombycis* and *N. spodopterae* than to PX2. Comparison of the identities of the rRNA domains and of the other three genes showed a high divergence in the sequences of the rRNA spacer regions (ITS and IGS). This is consistent with the hypothesis that PX2, if not PX1, might represent a new *Nosema* species.
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Keywords: Phylogenetic analysis; *Plutella xylostella*; *Nosema* spp.; rRNA; Tubulin gene; RPB1

1. Introduction

Microsporidian classification is based on life cycle stages and ultrastructural characteristics, as well as on microsporidium-host species relationships (Sprague, 1977). Thus, three closely related microsporidian genera, *Nosema*, *Vairimorpha*, and *Thelohania*, can be distinguished based on the occurrence of octosporous sporogony and a dimorphic karyotype (Linde et al., 1988; Pilley, 1976; Tanada and Chang, 1962; Tanada, 1962). However, taxonomic determinations based on these criteria alone can sometimes be problematic. For example, although the microsporidium *Vairimorpha imperfecta* usually exhibits octosporous sporogony, this character is abortive when *V. imperfecta* infects the diamondback moth *Plutella xylostella* (Jungen,

1996; Nahif and Jungen, 1998; Canning et al., 1999). Furthermore, based on its SSUrRNA (small subunit rRNA) sequence, *V. imperfecta* clusters in a clade with *Nosema* spp. from Lepidoptera rather than in the clade containing the *Vairimorpha* type species, *V. necatrix* (Baker et al., 1994; Canning et al., 1999). Bearing these considerations in mind, in the present paper we attempt to make a taxonomic determination for a new microsporidian isolate (designated PX2) by taking into account both morphological characters and gene sequence data.

The PX2 microsporidian isolate was originally collected from a moribund larva of *P. xylostella* in central Taiwan. After observing that the PX2 spores were similar in shape to those of *N. spodopterae*, and also to those of another *Nosema* isolate, PX1 (Tsai et al., 2003), we proceeded to analyze the phylogenetic relationships between PX2, PX1, and two *Nosema* species found in Taiwan (*N. bombycis* and *N. spodopterae*) by comparing their rRNA sequences and the sequences of three other genes. We also investigated

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relatedness to other microsporidian species for which data were available.

2. Materials and methods

2.1. Microsporidian isolates, host culture, and Western blotting

A moribund larva of *P. xylostella* with microsporidiosis was collected from Taichung in central Taiwan. The larva was homogenized and mature spores of the microsporidian isolate, designated, PX2, were harvested. Following the procedure described by Tsai et al. (2003) for the *Nosema* isolate PX1, the PX2 spores, and also spores of PX1 (from a moribund *P. xylostella* larva in Taipei) and of *N. spodopterae* (from laboratory-reared *Spodoptera litura*) were separately introduced into third instar larvae of laboratory-reared *S. litura* by oral inoculation. Then, after culturing and harvesting, spore bands were collected, purified and stored in Tris–EDTA buffer at 4 °C.

Subsequently, soluble polypeptides of the PX1, PX2 and *N. spodopterae* spores were prepared, separated by 12.5% SDS–PAGE and subjected to Western blotting with rabbit-anti-*S. litura Nosema* spore antiserum, as described in Tsai et al. (2003).

2.2. Sequencing and phylogenetic analysis of PX1 and PX2 genes

Primers used in the sequencing of the complete PX1 and PX2 rRNA genes were designed based on fragments amplified by the LSUF/ILSUR primer set (Tsai et al., 2003). These Primers were: Px-L1: GAGTACGATTGCTTGGT AGTGC; Px-LR1: GCCCCAGCCAAACTCCCC; Px-L3: CGTGTTACAACGTACCTACC; Px-LR3: GTTACTAC CACCAAGATCTGC; Px-L4: CGTACCTATACCGCA TCAGG; Px-LR4: ATACTCAACTGTCCTAAGAGAC;

TL2F: GACGTGCAAATCGATGGTG; TL2R: GTCAT AGTTACTCCCGCCG. Other previous published primers were used to amplify the alpha- and beta-tubulin and RPB1 (DNA dependent RNA polymerase II largest subunit) genes (Tsai et al., 2003; Vossbrinck et al., 1993; Gatehouse and Malone, 1998; Keeling and Doolittle, 1996; Keeling et al., 2000; Cheney et al., 2001). PCR amplification of the respective spore suspensions (2×10^8 spores in 0.25 ml TE buffer) was as described in previous papers (Huang et al., 2004; Tsai et al., 2005). Subcloning of the resulting dsDNA fragments of about 1.2 kb was performed using the pGEM-T Easy Vector System (Promega), after which both DNA strands were sequenced on an automated DNA sequencer (DNA sequencer 377, Applied Biosystems).

The SSUrRNA gene sequence of PX2 was compared with that of PX1 and with the SSUrRNAs of microsporidia from other lepidopteran hosts. Sequences were obtained from GenBank (Supplementary Data A) and aligned with the CLUSTAL X program (Thompson et al., 1997). LSUr-RNA gene sequences were also compared, as well as the conserved regions of the three other genes (alpha-tubulin, beta-tubulin, and RPB1). Phylogenetic analyses based on the resultant alignments were then constructed using the maximum likelihood (ML), the neighbor-joining algorithm (NJ), and the maximum parsimony method (MP), as implemented by PAUP 4.0b4a (Swofford, 1998). The nucleotides of tubulin and RPB1 genes were used to construct the phylogenetic trees. All the bases were used and no corrections were made for rate variations. The NJ algorithm was run using the Kimura-2 parameter, and for ML and MP, the full heuristic method was used. One thousand bootstrap replications and one hundred character resamples were generated to test the robustness of the trees.

Phylogenetic analysis of multiple genes included rRNA (SSUrRNA and LSUrRNA) and other genes (alpha-tubulin, beta-tubulin, and RPB1). *Schizosaccharomyces prombe* was used as an out-group. All sequences were aligned using

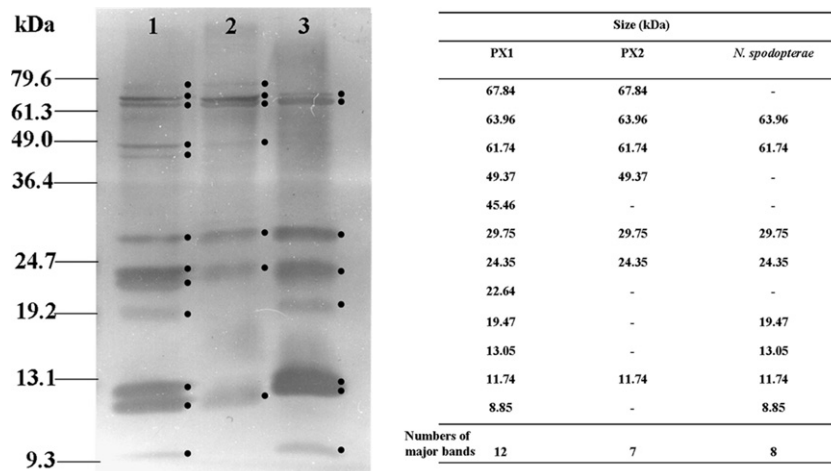| kDa	Size (kDa)		
	PX1	PX2	N. spodopterae
	67.84	67.84	-
	63.96	63.96	63.96
	61.74	61.74	61.74
	49.37	49.37	
	45.46	-	-
	29.75	29.75	29.75
	24.35	24.35	24.35
	22.64	-	-
	19.47	-	19.47
	13.05	-	13.05
	11.74	11.74	11.74
	8.85	-	8.85
Numbers of major bands	12	7	8

Fig. 1. Western-blot pattern of the spore-soluble polypeptides of three microsporidia with rabbit anti-*Nosema spodopterae* spore antiserum. Lane 1: PX1; Lane 2: PX2; Lane 3: *N. spodopterae*. Molecular weights of markers are given in kDa on the left, and the molecular weights of the major detected bands (indicated by spots) are given in the panel on the right.

the "Clustal X 1.18" program (Thompson et al., 1997). The resulting alignment was manually edited using the "Gene-Doc" program (Nicholas et al., 1997). The multiplex genes (SSUrRNA and LSUrRNA for Fig. 2d; alpha-tubulin, beta-tubulin, and RPB1 for Fig. 2h) were combined for phylogenetic analysis. The multiplex gene phylogenetic analysis was performed using Bayesian inference (Rannala and Yang, 1996). The best-fitting model of DNA substitution was selected by the Akaike information criterion (AIC) using the "MrMODELTEST version 2.2". The Bayesian analysis was performed using "MrBayes version 3.1.2" (Huelsenbeck and Ronquist, 2001). Metropolis-coupled Markov chain Monte Carlo analyses were run with four chains (one cold chain and three heated chains). Anal-

yses were initiated with random starting trees, then run for 1×10^6 generations and were sampled every 100 generations. The burn-in period discarded 100,000 generations. Posterior clade probabilities were used to assess nodal support. Tree topology represented on the 50% majority-rule consensus trees.

3. Results

3.1. Soluble protein profiles

Fig. 1 shows the protein patterns of the two *P. xylostella* microsporidian isolates, PX1 and PX2, and of *N. spodopterae* after reaction with anti *N. spodopterae* spore antiserum.

a

Tubulin and RPB1 identities

	PX2			Ns			Nb		
	Alpha-tubulin	Beta-tubulin	RPB1	Alpha-tubulin	Beta-tubulin	RPB1	Alpha-tubulin	Beta-tubulin	RPB1
PX1	96(41)	96(42)	96(43)	97(25)	97(27)	96(42)	97(30)	98(15)	98(23)
PX2				97(31)	96(39)	95(53)	97(39)	97(32)	96(43)
Ns							98(21)	98(17)	96(49)

rRNA identities

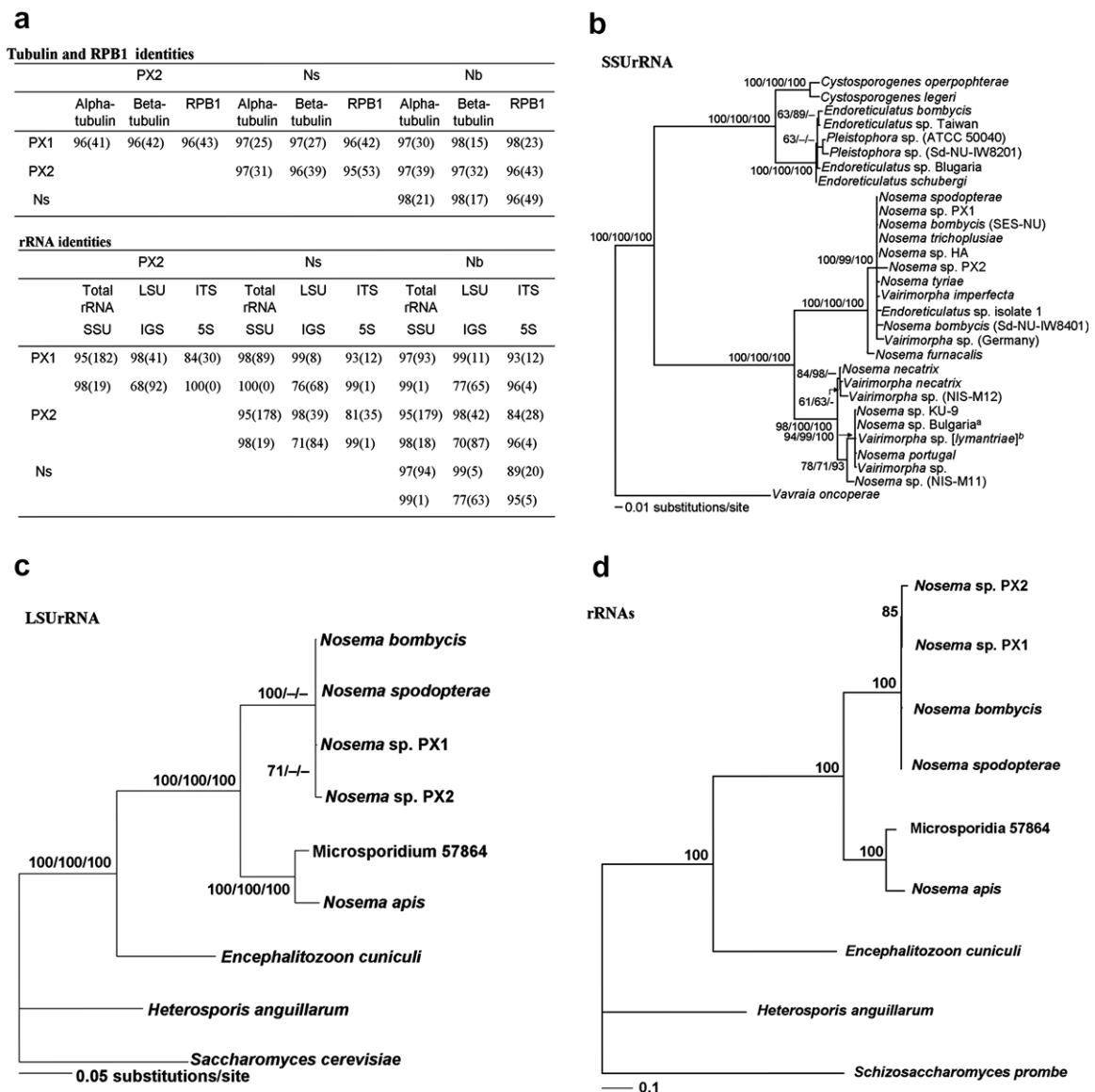|	PX2			Ns			Nb		
	Total rRNA	LSU	ITS	Total rRNA	LSU	ITS	Total rRNA	LSU	ITS
	SSU	IGS	5S	SSU	IGS	5S	SSU	IGS	5S
PX1	95(182)	98(41)	84(30)	98(89)	99(8)	93(12)	97(93)	99(11)	93(12)
	98(19)	68(92)	100(0)	100(0)	76(68)	99(1)	99(1)	77(65)	96(4)
PX2				95(178)	98(39)	81(35)	95(179)	98(42)	84(28)
				98(19)	71(84)	99(1)	98(18)	70(87)	96(4)
Ns							97(94)	99(5)	89(20)
							99(1)	77(63)	95(5)

b SSUrRNA

c LSUrRNA

d rRNAs

Fig. 2. Gene identities (%) and phylogenetic analysis of the microsporidian rRNA, alpha- and beta-tubulin, and RPB1 genes. (a) Gene identities and the number of base changes between species. The gene identities in the upper table are based on partial (conserved) sequence data for alpha-tubulin, beta-tubulin, and RPB1, and the rRNA gene identities in the lower table represent the % identity of the entire rRNA sequence, LSUrRNA, ITS, SSUrRNA, IGS, and 5S. Numbers in parentheses show the number of base changes between species. Nb, *Nosema bombycis*; Ns, *Nosema spodopterae*; PX1, *Nosema* sp. PX1; PX2, *Nosema* sp. PX2. (b, c, e, f, g) Phylogenetic trees constructed by the maximum likelihood method for SSUrRNA, LSUrRNA alpha-tubulin, beta-tubulin, and RPB1, respectively. The bootstrap values are ML/MP/NJ. (d, h) Bayesian analysis for rRNAs and other genes.
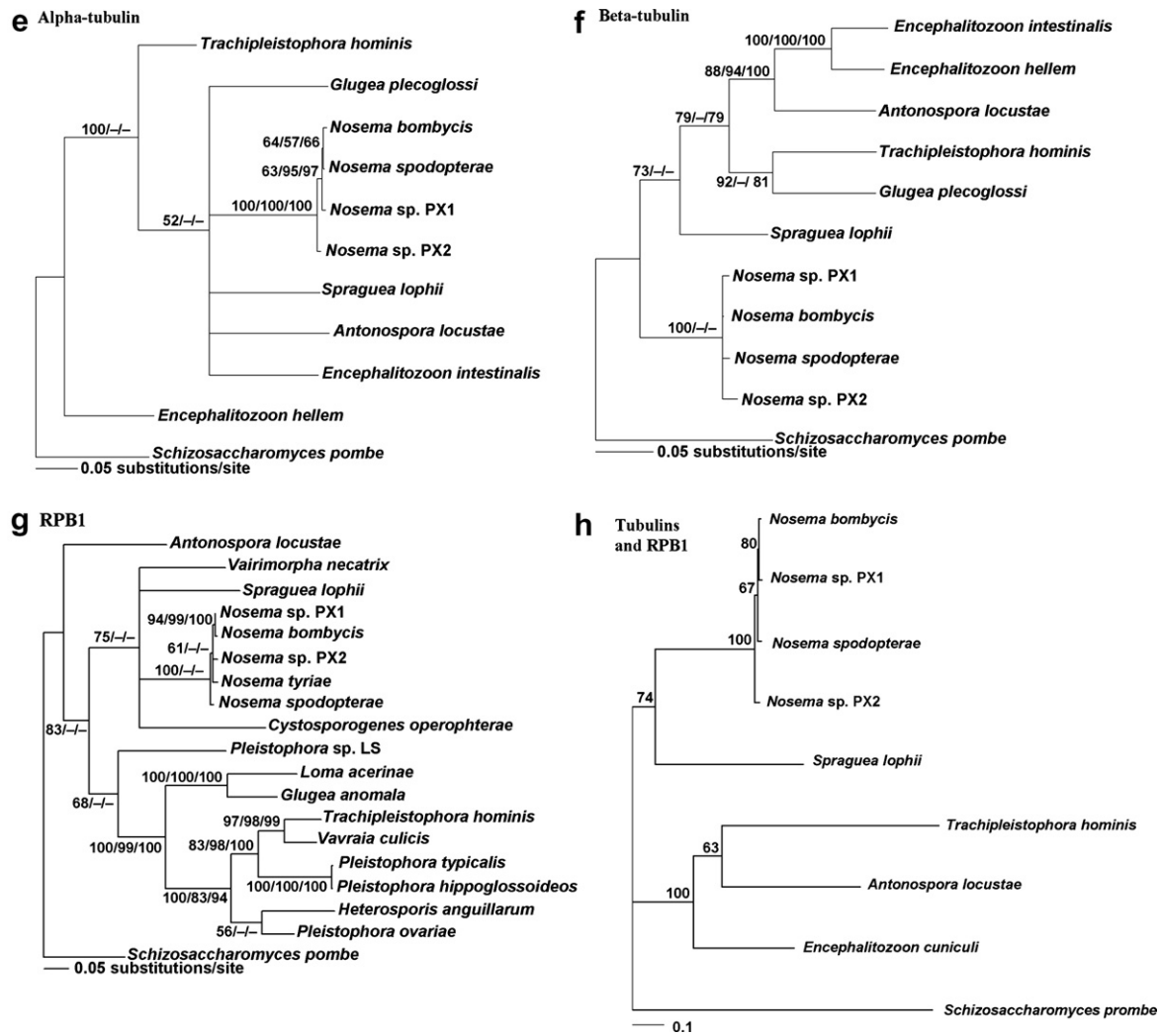
Fig. 2. (*continued*)

A total of 12 major bands were found in PX1. Seven of these bands were detected in PX2, and eight in *N. spodopterae*.

3.2. The entire sequence and organization of PX1 and PX2 rRNAs

Although the SSUrRNA domain for PX1 has already been sequenced (Tsai et al., 2003; GenBank Accession No. AF238239), here we sequenced the entire length of the PX1 and PX2 rRNA genes for the first time (PX1: 4309 bp; Gen-Bank Accession No. AY960986; PX2: 4305 bp; AY960987). The organization of the rRNA of both PX1 and PX2 was 5′-LSU-ITS-SSU-IGS-5S-3′, which is the same as that of *N. bombycis* (AY259631; Huang et al., 2004) and *N. spodopterae* (AY747307; Tsai et al., 2005). (We note that this organization, which is the reverse of the used 5′-SSU-ITS-LSU-IGS-5S-3′) organization found in Microspora, is a unique characteristic of all members of the *Nosema* genus that have so far sequenced, with the sole exception of *N. apis* (Huang et al., 2004). The lengths of the domains of the PX1 and PX2 rRNA genes are, respectively, as follows:

LSU: 2498, 2493; ITS: 185, 180; SSU: 1232, 1232; IGS: 279, 285; 5S: 115, 115 bp. Fig. 2a shows the percentage sequence identities of the rRNA genes of these four microsporidia, as well as for their alpha-tubulin (1202 bp), beta-tubulin (1162 bp) and RPB1 genes (1329 bp for PX1; 1330 bp for PX2).

3.3. Phylogenetic analyses

Based on the SSUrRNA gene sequences, the maximum likelihood (ML) method (Fig. 2b) and the neighbor joining (NJ) and maximum parsimony (MP) methods (NJ and MP trees are provided as Supplementary Data B) all found very similar phylogenetic relationships among the *Nosema/Vairimorpha* species and isolates. PX1 and PX2 were in a clade together with *N. bombycis* and *N. spodopterae*. This clade consisted of two *Vairimorpha* species, *V. imperfecta* (AJ131645) and a German isolate (AF124331) from *P. xylostella*. On the basis of the LSUrRNA gene sequences, ML grouped PX1 and PX2 together (Fig. 2c), while NJ and MP found that PX2 was unique and grouped PX1 together with *N. bombycis* and *N. spodopterae* (Data not shown). All

three methods located *N. apis* (U97150) and Microsporidium 57864 (U90885) in another clade, although this clade was close to the four other *Nosema* species/isolates. Base on the multiple gene analysis of small and large subunit rRNA, (Fig. 2d), Px1 and PX2 were also grouped together.

Based on the alpha-tubulin gene sequences, all three methods found that PX2 was unique, and grouped the three other species together. ML and MP found that *N. bombycis* was more closely related to *N. spodopterae* than to PX1 (Fig. 2e). Based on the beta-tubulin gene sequences, the ML method grouped PX2 with the other *Nosema* spp. (Fig. 2f), while the NJ and MP methods found that PX2 was unique (data not shown). Based on the RPB1 gene sequences, all three methods yielded similar results: PX1 was grouped together with *N. bombycis* and was more closely related to PX2 than to *N. spodopterae*; *N. spodopterae* was unique (Fig. 2g). Bayesian analysis for tubulins and RPB1 (Fig. 2h) showed that PX1 was grouped with *N. bombycis*, but PX2 was unique.

Thus, in contrast to the lack of resolution obtained based on the SSUrRNA and LSUrRNA genes, the phylogenetic trees of alpha-tubulin, beta-tubulin, and RPB1 were much better able to distinguish the PX2 isolate from others.

4. Discussion

Although the data presented here are not conclusive, they are consistent with the hypothesis that PX2 represents a new *Nosema* species. In support of this, Fig. 1 suggests that PX2 is a new serotype, while the phylogenetic trees (Fig. 2) suggest that PX2 is unique on the basis of the alpha-tubulin and RPB1 sequences. The Bayesian analysis provided additional evidence for PX2's uniqueness. In addition, the sequence identity data in Fig. 2a show high divergence for the ITS and IGS regions, and for these sequences PX2 had the lowest identities. Based on the data in Fig. 2, it is hard to distinguish the closest relative to PX2 between PX1, *N. spodopterae* and *N. bombycis* are all about equally distant. SSUrDNA nucleotide differences, for example, are all similar (19, 19 and 18, respectively; see Fig. 2a). The number of nucleotide differences may be large enough to at least suggest that PX2 is a separate species, but as Canning et al. (1999) have pointed out, while the sequence of SSUrRNA and other highly conserved genes may be useful for genus determination, but they are probably not good for species identification. Unfortunately, for the less conserved genes (alpha- and beta-tubulin, RPB1) only partial sequences are presently available, and although it is likely that they will be better candidates than rRNA genes for species identification, they will first have to be completely sequenced and analyzed in a more comprehensive fashion. In addition, Bayesian analysis for multiplex gene could be a possible approach for better resolution.

In conclusion, although PX2 may well represent a new *Nosema* species, more data, including morphological evidence, will need to be gathered before this determination can be confirmed. Meanwhile, for PX1, the data presented here suggest that it is closely related to *N. bombycis* and *N. spodopterae*, which is consistent with Tsai et al. (2003), who concluded that PX1 is probably a sub-species of *N. bombycis*.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jip.2006.11.008.

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