The characterization of microsporidian isolates (Nosematidae: *Nosema*) from five important lepidopteran pests in Taiwan

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Abstract

Microsporidian isolates from five lepidopteran pests—*Spodoptera litura*, *Spodoptera exigua*, *Helicoverpa armigera*, *Plutella xylostella*, and *Pieris* spp.—were compared by spore morphology, infectivity to *S. litura*, Western-blot banding patterns, the sequences of small subunit rRNA gene (SSUrRNA sequence), and random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). All the isolates could infect experimentally and multiply in the larvae of *S. litura*. The *S. exigua* isolate showed the highest virulence to the larvae of *S. litura* while the *P. xylostella* isolate showed the lowest. No significant differences either in spore morphology or in SSUrRNA sequences of these isolates were found. The SSUrRNA sequences of these isolates confirmed they are members of the genus *Nosema*. Based on the result of Western-blot hybridization with the rabbit anti-*Nosema spodopterae* spore antiserum, three serotypes could be distinguished: *N. spodopterae* (*S. litura* isolate) and *Pi. spp.* isolate; *S. exigua* and *H. armigera* isolates; and *P. xylostella* isolate. The amplicons of RAPD-PCR with 60 primers yielded clear patterns that were selected and used for identification and also for phylogenetic analysis of these five isolates. Based on analysis by the computer, isolates could be clearly divided into three groups that were coincident with the serotypes; therefore we suggest that *N. spodopterae* and isolates of *Pi. spp.*, *S. exigua*, and *H. armigera* are more closely related in phylogenesis. In addition, in the amplification with the *Nosema bombycis* specific primer set, only DNAs from *P. xylostella* isolate and *N. bombycis* yielded amplicons. Therefore, we suggest that four isolates, excluding the *P. xylostella* isolate, are *N. spodopterae*, and the taxonomic position of *P. xylostella* isolate needs to be elucidated. © 2003 Elsevier Science (USA). All rights reserved.

Keywords: *Nosema* spp.; Infectivity; RAPD; SSUrDNA; Western-blot hybridization

1. Introduction

Microsporidia have been usually considered primitive, without mitochondria, eukaryotic protists that are obligate intercellular parasites. They are very common in insects and in the intimate parasites of insect hosts, well adapted in pathogenicity, transmission, ecology, and resistance to the defense system of their hosts. Recently, molecular biological and genomic studies on microsporidia have concentrated on many unusual features, especially mitochondrial genes. The accumulation

of molecular evidence has shown that microsporidia are not really the most primitive eukaryotes, in fact, highly specialized fungi (Keeling and Fast, 2002). Insects in nearly all taxonomic orders are susceptible to this pathogen and over half of the susceptible insect hosts belong to one of two orders, Lepidoptera and Diptera. Several species of microsporidia play an important role in regulating insect populations (Tanada and Kaya, 1993). The members of genus *Nosema* are often considered the most important and widely distributed group of microsporidia.

The life cycle stages and parasite–host relations of microsporidia possess many unique ultrastructural characteristics and are critical to taxonomic determination

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of microsporidia genera and species. The taxonomy of microsporidia usually follows the system developed by Sprague (1977). Since many microsporidia are considered to have a wide host range (Tanada and Kaya, 1993), the life cycles and parasite–host relationships may be inadequate as taxonomic criteria in some cases, especially in species determination. In such cases, more reliable tools are needed for diagnosing and identifying intra- or interspecies. Protein and serologic analyses (Irby et al., 1986; Ke et al., 1990; Streett and Briggs, 1982) and the rRNA gene sequences (Baker et al., 1994; Malone and McIvor, 1996; Pieniazek et al., 1996; Visvesvara et al., 1994; Vossbrinck et al., 1987; Vossbrinck et al., 1993) of microsporidia have been commonly used for this purpose. In addition to rRNA gene sequencing, random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has emerged as a fast and sensitive technique for distinguishing closely related organisms. This method had been used to assess the polymorphism of closely related microsporidian isolates of *Encephalitozoon* spp. obtained from different hosts (Mathis et al., 1996, 1997).

Microsporidian isolates from five important lepidopteran pests—the tobacco cutworm, *Spodoptera litura*; the beet armyworm, *Spodoptera exigua*; the corn earworm, *Helicoverpa armigera*; the diamondback moth, *Plutella xylostella*, and two cabbage worms, *Pieris* spp. (*Pieris rapae crucivora* and *Pieris canidia sordida*)—were collected in Taiwan. Considered the most economically devastating, these five lepidopteran pests have steadily increased their infestation of cruciferous vegetables in Taiwan and are frequently found even in the same crops. The microsporidian isolate of *S. litura* has been identified and named *Nosema spodopterae* (Hsu et al., 1992) while the taxonomic positions of other isolates are still unclear. It is reasonable to suspect that they may belong to the same species or closely related species. In an attempt to clarify their taxonomic position and understand their relationship, several characteristics—including morphology, infectivity to *S. litura*, protein, and Western-blot banding profiles—the sequences of small subunit rRNA (SSUrRNA) gene and RAPD-PCR of five isolates were examined and analyzed in this paper.

2. Materials and methods

2.1. Microsporidian isolates and host culture

Microsporidian isolates were collected from five lepidopteran pests: *S. litura* (*N. spodopterae* abbreviated as SL); *S. exigua* (SE); *H. armigera* (HA); *P. xylostella* (PX); and *Pieris* spp. (PS). The spores of all isolates were harvested and propagated in the larvae of *S. litura*. The surrogate hosts were reared on an artificial diet (Koyama and Kamano, 1976) in a growth chamber at

$26 \pm 2^\circ\text{C}$ and 75–80% relative humidity. The photoperiodic regime was 12L/12D. The surrogate hosts were inoculated with different concentrations (1×10^3 , 1×10^4 , and 1×10^5) of each isolate's spores by a spore-contaminated artificial diet. Moribund larvae were collected and homogenized in Tris–EDTA buffer (TE; 10 mM Tris, 1 mM EDTA, pH 7.5). The homogenates were filtered through three layers of cheesecloth and centrifuged at 1000g for 10 min. The pellets were resuspended in TE buffer and the spores were purified by 90% Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation at 30,000g for 30 min (Tsai and Wang, 2001; Tsui et al., 1988). The spores formed a white band close to the bottom of the centrifugal tube. The spore band was collected and washed several times by TE buffer. The purified spores of type species, *Nosema bombycis*, were the gift of Dr. Sugimoto, MAFF GENE Bank of NIAS (National Institute of Agrobiological Science), Japan. They were stored at 4°C .

2.2. Experimental infection to *S. litura* larvae

Three doses, 1×10^3 , 1×10^4 , and 1×10^5 spores, of each isolate and *N. bombycis* were dispensed onto the upper surface of an artificial diet ($5 \times 5\text{ mm}^2$). Control insects were supplied with distilled water on an artificial diet only. Newly third instar larvae (5-day-old) of *S. litura* were starved in 30-well plastic plates for 4–6 h before experimental infection. The larvae were then supplied or not supplied with a spore-contaminated artificial diet. Mortality of the larvae was recorded and calculated by probit analysis and the cadavers were collected and stored at 4°C . The spore yield of the infected larvae with each isolate at 30 days post-inoculation was also calculated. The spores were then purified by Percoll gradient centrifugation and stored at 4°C .

2.3. Preparation of anti-*N. spodopterae* spore antiserum

The anti-*N. spodopterae* spore antiserum was prepared in a male New Zealand White rabbit weighing about 1.5 kg. One billion spores in 1 ml phosphate buffered saline (PBS, pH 7.3) were shaken with 0.5 g glass beads (0.5 mm diameter) to disrupt them (Tsai et al., 2002). The lysed spores were used as inoculum. The inoculum was emulsified with 0.5 ml adjuvant. Freund's complete adjuvant was used for the first immunization and Freund's incomplete adjuvant for the following immunizations. After three boosts, the rabbit serum was used as antiserum for detection.

2.4. SDS–polyacrylamide gel electrophoresis and Western-blot hybridization

The spore-soluble polypeptides were prepared from the homogenates of 4×10^8 spores with glass beads, as

described above. The homogenates were centrifuged at 15,000g for 5 min, and the supernatants were obtained by boiling them for 10 min in an equal volume of 2× SDS sample buffer (10% β-mercaptoethanol, 4% SDS, and 0.01% bromophenol blue in 0.1 M Tris–HCl buffer, pH 6.8). The polypeptides of the microsporidian isolates samples were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with 12.5% acrylamide gel (Laemmli, 1970). The polypeptide bands were transferred onto the nitrocellulose membranes by SEMI-PHOR (Hoefler). The membranes were blocked with 3% skim milk in Tris-buffered saline (TBS; 20 mM Tris, 0.5 M NaCl, pH 7.5) for 1 h, washed with TBS containing 0.05% Tween 20 (TTBS) for 3 min, and then incubated with the rabbit anti-*S. litura* *Nosema* spore antiserum which were diluted 100-fold for 1 h. After triple washing with TTBS (3 min each), the membranes were incubated with a 1:2500 dilution of peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. After three more washing with TTBS and one with TBS, the membranes were then incubated with a color-developing substrate solution. Hydrogen peroxide and 4-chloro-1-naphthol were used as substrate and chromogen, respectively.

2.5. Sequencing of small subunit rRNA

Spore suspensions (2×10^8 spores in 0.25 ml TE buffer) mixed with equal volumes of zirconia/silica beads (0.1 mm diameter) in 10 × 75 mm glass tubes were shaken at maximum speed on a vortex mixer for 1 min

(modification of Undeen and Cockburn, 1989; Tsai et al., 2002). The homogenates were incubated with proteinase K for 1 h at 56 °C. DNA was extracted in phenol/chloroform, precipitated with ethanol, and then amplified by polymerase chain reaction (PCR). A primer set (18f/1537r: 5'-CACCA GGTG ATTCT GCC-3'/5'-TTATG ATCCT GCTAA TGGTT C-3') was used for amplification of the microsporidian SSUrRNA coding region (Vossbrinck et al., 1987, 1993). The DNA template (100 ng) of each isolate was mixed in a 100 μl reaction mixture containing 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 200 mM of each dNTP, 100 pmol of each primer, and 2.5 U Taq DNA polymerase (Promega, Madison, WI). The amplification was performed in an AG-9600 Thermal Station (Biotronics Corp.) for 40 cycles, each with the following profile: 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min. A 10 μl aliquot from each reaction was run on a 1.0% agarose gel to visualize the PCR product. The DNA fragment of about 1.2 kb was then subcloned into the *Sma*I site of pUC18 vector, and both DNA strands of cloned DNA fragments were sequenced on an automated DNA sequencer (DNA sequencer 377, Applied Biosystems).

2.6. Phylogenetic analysis

The microsporidian SSUrRNA gene sequences of the isolates and the other lepidopteran host, from GenBank (Table 1) were aligned with the CLUSTAL X program (Thompson et al., 1997). The sequence from *Vavraia oncoperae* (Accession No. [X74112](#)) was an outgroup.

Table 1

The DNA sequences of microsporidia derived from lepidopteran hosts used for SSUrDNA phylogenetic analysis

Organism	Host	GenBank Accession No.
<i>Endoreticulatus schubergi</i>	<i>Lymantria dispar</i>	L39109
<i>Nosema bombycis</i> (SES-NU)	<i>Bombyx mori</i>	D85503
<i>Nosema furnacalis</i>	<i>Ostrinia furnacalis</i>	U26532
<i>Nosema necatrix</i>	<i>Pseudaletia unipuncta</i>	U11051
<i>Nosema portugal</i>	<i>Lymantria dispar</i>	AF033316
<i>Nosema</i> sp. (NIS-M11)	<i>Bombyx mori</i>	D85501
<i>Nosema bombycis</i> (Sd-NU-IW8401)	<i>Spodoptera depravata</i>	D85504
<i>Nosema</i> sp. HA	<i>Helicoverpa armigera</i>	AF238240
<i>Nosema</i> sp. KU-9	A noctuid moth	AF141130
<i>Nosema</i> sp. SL	<i>Spodoptera litura</i> , <i>S. exigua</i> , <i>Plutella xylostella</i> , and <i>Pieris</i> spp.	AF238239
<i>Nosema trichoplusia</i>	<i>Trichoplusia ni</i>	U09282
<i>Nosema tyriae</i>	<i>Tyria jacobaeae</i>	AJ012606
<i>Pleistophora</i> sp. (ATCC 50040)	<i>Agrotis exclamationis</i>	U10342
<i>Pleistophora</i> sp. (Sd-NU-IW8201)	<i>Spodoptera depravata</i>	D85500
<i>Vairimorpha imperfecta</i>	<i>Plutella xylostella</i>	AJ131645
<i>Vairimorpha lymantriae</i>	<i>Lymantria dispar</i>	AF033315
<i>Vairimorpha lymantriae</i> (2)	<i>Lymantria dispar</i>	AF141129
<i>Vairimorpha necatrix</i>	<i>Malacosma americanum</i>	Y00266
<i>Vairimorpha</i> sp.	<i>Lymantria dispar</i>	L39114
<i>Vairimorpha</i> sp. (Germany)	<i>Plutella xylostella</i>	AF124331
<i>Vairimorpha</i> sp. (NIS-M12)	<i>Bombyx mori</i>	D85502
<i>Vavraia oncoperae</i>	<i>Wiseana</i> sp.	X74112

Phylogenetic analysis based on the resultant alignment was using neighbor-joining algorithm methods for distance analysis (Kimura 2-parameter) with PAUP 4.0b8 (Swofford, 1998). One-thousand bootstrap replicates and one-hundred characters resampling were generated to test the robustness of the tree.

2.7. Random amplified polymorphic DNA

RAPD-PCR amplifications were performed according to the standard technique described by Williams et al. (1990) with minor modifications. A total of 100 primers of five Operon 20-primer sets—OPA, OPAC, OPBD, OPO, and OPU (Operon Technologies, Alameda, CA)—were used as RAPD primers. The total volume of the reaction mixture was 25 μ l, containing 10 ng DNA, 50 mM KCl, 10 mM Tris–Cl (pH 9.0 at 25 °C), 0.1% Triton X-100, 2.0 mM MgCl₂, 0.2 mM each dNTP, 0.2 μ M primer, and 0.625 U Taq DNA polymerase. The samples were incubated in 0.2 ml tubes in an AG-9600 Thermal Station for 2 min at 94 °C (the initial denaturation step) and then for 45 cycles consisting of 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min. Following these cycles the samples were incubated at 72 °C for 7 min and then held at 4 °C. RAPD polymorphism was analyzed on 1.6% ethidium bromide-stained agarose gels and photographed using the Eagle-Eye II photo-documentation system (Stratagene). Sixty of 100 primers were able to yield clear amplicon bands, and these were used for analysis. Selected amplicons were number-coded and scored as present or absent for each isolate. The number of shared bands was used for the similarity measure and the consistently amplified DNA fragments were used to produce a pairwise matrix using the Jaccard similarity coefficients. The coefficients were employed to construct dendrograms based on the Unweighted Pair-Group Method with Arithmetic Averages (UPGMA) applying software from the NTSYS-pc v. 2.01b (Rohlf, 1997).

2.8. Specific primers PCR

The KAI01N (5'-GTA GTA GAG ACC CAA CTA TC-3') and KAI02N (5'-ACT GTT CAG ATA TGG

TCC TTA TCG-3') primers were modified from a sequence of a putative pseudogene of small subunit *N. bombycis* SES-NU rRNA (Kawakami et al., 1995). The PCR conditions were described above and the amplicon patterns were photographed using the Eagle-Eye II photo-documentation system (Stratagene).

3. Results

3.1. Morphology and pathogenicity of the isolates

Spore sizes of the five isolates were $4.00 \pm 0.17 \times 1.90 \pm 0.12 \mu\text{m}$ (SL), $3.98 \pm 0.25 \times 1.90 \pm 0.12 \mu\text{m}$ (SE), $4.03 \pm 0.26 \times 1.89 \pm 0.14 \mu\text{m}$ (HA), $3.96 \pm 0.21 \times 1.88 \pm 0.10 \mu\text{m}$ (PX), and $4.01 \pm 0.21 \times 1.88 \pm 0.11 \mu\text{m}$ (PS), respectively. No significant difference among these isolates was indicated by Duncan's multiple range test.

The third instar larvae of *S. litura* are susceptible to all microsporidian isolates. The median lethal doses (LD₅₀) of the five isolates to third instar larvae of *S. litura* were 1.78×10^3 (SL), 1.10×10^3 (SE), 4.28×10^3 (HA), 2.88×10^3 (PX), and 1.29×10^3 (PS) spores/larva at 30 days post-inoculation, respectively (Table 2). The cumulative mortality of the larvae is shown in Fig. 1. Apparently, SE isolate showed the highest virulence to the larvae of *S. litura* (formed an S-shaped curve), and PX isolate showed the lowest. Except SE isolate, the high spore yields of the infected *S. litura* larvae were obtained at a high dosage of 1×10^5 (Table 2). Therefore, the larvae of *S. litura* were to be a substitute for these five isolates in this study.

3.2. Western-blot banding patterns

The result of Western-blot hybridization with rabbit anti *N. spodopterae* spore antiserum is shown in Fig. 2. The major bands were found at ranges from 72 to 49 kDa, 44, and 26 kDa. Based on the banding patterns, three serotypes, SL/PS, SE/HA, and PX could be distinguished. The patterns of SL/PS type (lanes 1 and 5) contained six bands: 72, 60, 54, 50, 44, and 26 kDa (Fig. 2, lanes 1 and 5). In addition to the six bands of SL/PS type, 67 kDa, was found in SE/HA type (Fig. 2, lanes 2

Table 2
Infectivity of five isolates to the third instar larvae of *S. litura* at 30 days post-inoculation

<i>Nosema</i> isolates ^a	LD ₅₀ at the 30th day (spores/larva)	LT ₅₀ (days) ^b	Spores yield (spores/larva) ^b	Mortality rate (%) ^b
SL	1.78×10^3	14.81	7.16×10^8	98.88
SE	1.10×10^3	10.82	4.09×10^7	100.00
HA	4.28×10^3	18.93	1.17×10^9	91.01
PX	2.88×10^3	22.01	2.49×10^9	93.26
PS	1.29×10^3	16.35	1.78×10^8	95.56

^a Abbreviations of isolates from *S. litura* (SL), *S. exigua* (SE), *H. armigera* (HA), *P. xylostella* (PX), and *Pieris* spp. (PS, including *Pi. rapae crucivora* and *Pi. canidia sordida*).

^b At dosage 1×10^5 spores/larva.

Fig. 1. The cumulative mortality (%) of the third instar larvae of *Spodoptera litura* infected with microsporidian isolates (1×10^5 spores). Abbreviations of the isolates are SL, SE, HA, PX, and PS derived from *S. litura*, *S. exigua*, *H. armigera*, *P. xylostella*, and *Pieris* spp. (including *Pi. rapae crucivora* and *Pi. canidia sordida*) respectively.

Fig. 2. Western-blot hybridization of the spore-soluble polypeptides of five isolates with rabbit anti-*Nosema spodopterae* spore antiserum. Three serotypes can be distinguished: SL/PS (lanes 1 and 5), SE/HA (lanes 2 and 3), and PX (lane 4). Abbreviations of the isolates are SL, SE, HA, PX and PS derived from *S. litura*, *S. exigua*, *H. armigera*, *P. xylostella*, and *Pieris* spp. (including *Pi. rapae crucivora* and *Pi. canidia sordida*), respectively. Molecular weights are given in kDa on the left. Arrowheads show the unique bands of isolates.

and 3). PX type showed a most different pattern that contained two unique bands at 58 and 56 kDa (Fig. 2, lane 4) and lacked three bands: 67, 54, and 44 kDa.

3.3. SSUrDNA sequences

The amplified SSUrRNA gene region length of each isolate is 1232 bp. The sequences of four isolates (SL, SE, PX, and PS) are identical and only one base is different from HA isolate at position 821 from the 5'-end of the SSUrRNA gene. Both sequences were submitted to the GenBank, and the accession numbers were AF238239 (SL series) and AF238240 (HA). Phyloge-

netic analysis suggests that the microsporidia from lepidopteran can be divided into three distinct groups: the *Nosema*, *Vairimorpha*, and *Endoreticulatus* groups. The sequence of SSUrRNA gene of *Vavraia oncoperae* was used as an outgroup for this analysis (Fig. 3). The *Nosema* group consists of *N. bombycis* complex and *Nosema furnacalis*. The members of *N. bombycis* complex are the type species of genus *Nosema*, *N. bombycis*, most of the *Nosema* species, and two *Vairimorpha* species from *P. xylostella*. The *Vairimorpha* group consists of two subgroups, *Vairimorpha necatrix* complex, including the type species of genus *Vairimorpha*, *V. necatrix*, and *Vairimorpha lymantriae* complex, most members of which are derived from gypsy moth, *Lymantria dispar*, except for *Nosema* sp. KU-9, derived from a noctuid moth. *Nosema* sp. NIS-M11 is another *Nosema* species in *Vairimorpha* group, but it was considered a *Vairimorpha* species (Iguchi et al., 1997). The *Endoreticulatus* group consists of *Endoreticulatus schubergi* and two *Pleistophora* species from noctuid moths.

Fig. 3. Phylogenetic relationships among microsporidia divided from lepidopteran hosts based on SSUrDNA sequences. The tree was generated by the neighbor-joining method using Kimura two-parameter distances. The *Vavraia oncoperae* was used as an outgroup. Numbers next to the nodes refer to the bootstrap scores (%) in 1000 replicates. E, *Endoreticulatus*; N, *Nosema*; P, *Pleistophora*; V, *Vairimorpha*.

3.4. RAPD analysis

The efficiency of primers to generate a clear amplicon pattern after RAPD-PCR with isolate DNA was evaluated, and 60 primers were chosen and used for this study. Fig. 4 shows the representation of the amplicon patterns. Three distinct patterns could be distinguished. Based this, the primers were conveniently divided into: (1) nondiscrimination (8 primers): OPAC-01, -15, -19; OPO-03, -20 (Fig. 4A); and OPU-09, -10, -13; (2) discrimination among five microsporidia (3 primers): OPAC-09; OPO-10; and OPU-16 (Fig. 4B); and (3) partial discrimination consists of two subgroups: (I) three types (NS/PS, SE/HA, and PX, similar to serotypes; 11 primers): OPA-05, -08, -14, -15, -16; OPBD-11, -17, -18, -20 (Fig. 4C); and OPU-01, -17; (II) two types (PX and others not included in the second and third (I) groups; 38 primers): OPA-03, -04, -06, -07, -09, -12, -13, -17, -18; OPAC-03, -05, -07, -10, -12, -13, -14, -17, -18; OPBD-04, -05, -06, -07, -12, -14, -15; OPO-06, -13, -14; and OPU-02, -03 (Fig. 4D), -06, -08, -12, -14, -15, -18, -19, -20. In the phylogenetic analysis, the five isolates were clearly divided into three groups (Fig. 5): SL/PS,

Fig. 4. Four distinct amplicon patterns (A–D) of RAPD-PCR profiles for five microsporidian isolates with primer sets (OPA, OPAC, OPBD, OPO, and OPU) could be distinguished. The representative RAPD-PCR profiles for each pattern are shown: (A) nondiscrimination: the profile of OPU-20; (B) discrimination: five types: the profile of OPU-16; (C) partial discrimination: (I) three types (NS/PS, SE/HA, and PX); the profile of OPBD-20; and (D) partial discrimination: (II) two types (PX and others that are not including in B and C): the profile of OPU-03. M: Bio100 DNA ladder marker. Abbreviations of the isolates are SL, SE, HA, PX, and PS derived from *S. litura*, *S. exigua*, *H. armigera*, *P. xylostella*, and *Pieris* spp. (including *Pi. rapae crucivora* and *Pi. canidia sordida*), respectively.

Fig. 5. UPGMA dendrogram built from Jaccard's genetic distance matrix based on 248 RAPD fragments. Abbreviations of the isolates are SL, SE, HA, PX, and PS derived from *S. litura*, *S. exigua*, *H. armigera*, *P. xylostella*, and *Pieris* spp. (including *Pi. rapae crucivora* and *Pi. canidia sordida*), respectively.

Fig. 6. Agarose gel electrophoresis pattern of PCR amplification products of five microsporidian isolates by KAI01N/KAI02N primer-set showing that only PX (lane 4) and *N. bombycis* (lane 6) yield amplicons with 832 bp. Abbreviations of the isolates are SL, SE, HA, PX, PS, and BM derived from *S. litura*, *S. exigua*, *H. armigera*, *P. xylostella*, *Pieris* spp. (including *Pi. rapae crucivora* and *Pi. canidia sordida*), and *B. mori*, respectively. M: Bio100 DNA.

SE/HA, and PX. The isolates of SL and PS clustered with more than 93.5% similarity. The isolates of SE and HA clustered with more than 91% similarity. The similarity between SE/HA and SL/PS groups was 78.8%. PX isolate clustered with other isolates with about 21.3%.

3.5. Specific primers PCR

Fig. 6 shows KAI01N/KAI02N primer set produced an amplicon of PCR only with the DNA from spores of PX isolate and *N. bombycis*, but the others did not. Both amplicons are 832 bp (GenBank Accession No. AF310844). The sequences of both amplicons have only one base difference at position 48 (C) from the 5'-end, "T" in the *N. bombycis* pseudogene of *N. bombycis* SES-NUrRNA (GenBank Accession No. D14632) and "C" in that of PX isolate.

4. Discussion

Many reports have described the microsporidia from lepidopteran hosts. Okada (1970) has been recorded the microsporidian from *S. litura* in Japan, and Watanabe (1976) first described the *Nosema* species of *S. litura*.

This *Nosema* species did not have any harmful effects on the silkworm, *Bombyx mori*, even at high dosages for inoculation. Ke et al. (1990) indicated that *N. liturae* (*Nosema* species derived from *S. litura*) shared a common antigen determinant and were apparently closely related to other *Nosema* species from lepidopteran hosts. *S. exigua* was found infected by *Nosema* isolate, *Nosema* sp. Y9101, and this isolate was considered a strain of *N. bombycis* based on morphology and serology (Yasunaga et al., 1992). In Taiwan, the isolate from the larvae of *S. litura* was described and named *N. spodopterae* (Hsu et al., 1991, 1992). The massive microsporidian infections of the larvae of these lepidopteran pests, *S. litura*, *S. exigua*, *H. armigera*, *P. xylostella*, and *Pi. spp.*, occur in different seasons. The massive infections of *S. litura*, *S. exigua*, and *H. armigera* larvae occur in the fall, and those of *P. xylostella* and *Pi. spp.* larvae occur in the later spring and early summer in Taiwan. The microsporidian spores collected from these pests are morphologically similar, and they can infect experimentally and be multiplied in the surrogate host, the larvae of *S. litura*, while they are not susceptible to *N. bombycis* spores. In addition, the high similarity in the SSUrRNA sequences of five isolates implied that they are closely related in phylogeny and may even be the same species. In phylogenetic analysis, the SSUrRNA sequences of five isolates were aligned with other known microsporidian SSUrRNA gene sequences of species that infect the lepidopteran hosts (Fig. 3). The result leads us to confirm that these isolates are members of the *N. bombycis* complex and belong to the genus *Nosema*.

Both SSU and LSUrRNA genes are highly conserved among species while ITS is highly variable rRNA with a high rate of evolution. Canning et al. (1999) considered this highly conserved gene (SSUrRNA) useless in distinguishing between very closely related species, even those that can be distinguished on morphological criteria. Indeed, the SSUrRNA framework in each group is similar, but it can work on determination of an unknown species in high taxon and yet not work on identification of a closely related species (Fig. 3). We have found the sequences containing internal transcribed space (ITS) and partial of the 5'-end large subunit rDNA (LSUrDNA) from the *Vairimorpha* group (Accession Nos. AF141129, AF141130, AF033315, AF033316, and *V. necatrix* in Vossbrinck et al., 1993) but not from the *Nosema* group. Although a partial LSUrRNA gene sequence (Accession No. L28962) of *N. bombycis* (Baker et al., 1994) had been published, we could not find any ITS sequences of the *Nosema* group in the literature. Furthermore, only four microsporidian complete rRNA gene sequences—*Encephalitozoon cuniculi* (Peyretailade et al., 1998), *Microsporidium* 57864 (GenBank Accession No. U90885), *Nosema apis* (De Rijk et al., 1998), and *Heterosporis anguillarum* (Tsai et al., 2002)—have been published. It implied that a vast

difference of rRNA gene exists in the course of a high reduction of their genome and gene structure during evolution. Therefore, efforts to complete the rRNA gene sequences, especially in *Nosema* spp., will be a great contribution to understanding interspecies phylogenetic relationships. Among the isolates we collected, there were no significant difference in SSUrRNA sequences, but we believed that PX isolate was a distinct species. So far, we have carried out the complete sequence of *N. spodopterae* rRNA gene sequences (AY211390–AY211392), but those of other isolates, especially PX isolate and type species, *N. bombycis*, are also necessary to carry out what will be a great help in the *Nosema* species clarification.

Morphological characters and life cycle are the major criteria used to identify the microsporidia, but the species between genera *Nosema* and *Vairimorpha* are still ambiguous. In the phylogenetic analysis based on the SSUrRNA sequences (Fig. 3), several diversions, compared the identification in morphology, occur. The members of *N. bombycis* complex are very closely related, and they include two *Vairimorpha* species from *P. xylostella*. Canning et al. (1999) indicated that *Vairimorpha imperfecta* and the German isolate of *Vairimorpha* from the host *P. xylostella* were more closely related to the *Nosema* species from lepidoptera than to the *Vairimorpha* species. They chose to name it *Vairimorpha* because of the morphological characters. In addition, these two *Vairimorpha* species did not form octospores or sporophorous vesicles. This evidence hints at the need for a possible reexamination of the taxonomic position of these two species.

A similar situation appears in the *Vairimorpha* subgroup. The *V. lymantriae* complex consists of two *Nosema* species, *Nosema portugal* and *Nosema* sp. KU-9. Besides the *Nosema* KU-9, the others were all derived from gypsy moths, *Lymantria dispar*. *N. portugal* had been observed to have uninucleate meronts in merogonic cycles (Maddox et al., 1999), but it was not a characteristic typical of genus *Nosema*. *V. necatrix* complex contains *N. necatrix* too. The *Pleistophora* species from lepidopteran hosts are closely related to *Endoreticulatus schubergi*. Baker et al. (1995) suggested the *Pleistophora* sp. ATCC50040 (Accession No. U10342) was an *Endoreticulatus* species. These two *Pleistophora* species are not closely related to those that infect aquatic organisms.

The results of Western blot hybridization (Fig. 2) and Cluster analysis of RAPD (Fig. 4) characters showed that five *Nosema* isolates could be separated into three phenetic groups. In comparing RAPD profiles of the isolates, PX isolate shared only 21.3% of bands with others, indicating it was really genetically distinct from other isolates. Thus, it corroborated the results of Western blot hybridization. Furthermore, this conclusion was also confirmed by PCR with *N. bombycis*

specific primer set (KAI01N/KAI02N) (Kawakami et al., 1995). Only DNA from PX isolate and *N. bombycis* yielded amplicons (Fig. 6), and the amplicon sequences were identical, except for one nucleotide. Undoubtedly, PX isolate is a member of the genus *Nosema* but not of *N. spodopterae*. Indeed, the taxonomic position of *P. xylostella* isolate needs to be clarified in further study, especially the evidence of ultrastructure and other molecular markers.

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