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Phylogenetic relationships of three new microsporidian isolates from the silkworm, *Bombyx mori*

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Abstract

The pathogenicity, mode of transmission, tissue specificity of infection and the small subunit rRNA (SSU-rRNA) gene sequences of the three new microsporidian isolates from the silkworm *Bombyx mori* were studied. Out of the three, NIK-2r revealed life cycle features and SSU-rRNA gene sequence similar to *Nosema bombycis*, suggesting that it is *N. bombycis*. The other two, NIK-4m and NIK-3h, differed from each other as well as from *N. bombycis*. NIK-4m was highly pathogenic and did not show any vertical transmission, in accordance with the apparent lack of gonadal infection, whereas NIK-3h was less pathogenic and vertical transmission was not detected but could not be excluded. Phylogenetic analysis based on SSU-rRNA gene sequence placed NIK-3h and NIK-4m in a distinct clade that included almost all the *Vairimorpha* species and *Nosema* species that infect lepidopteran and non-lepidopteran hosts, while NIK-2r was included in a clade containing almost all the *Nosema* isolates that infect only lepidopteran hosts. Thus, we have presented molecular evidence that one of the three isolates is in fact the type species *N. bombycis*, while the other two isolates are *Vairimorpha* spp. There was distinct separation of microsporidian isolates infecting only lepidopteran hosts and those infecting lepidopteran and non-lepidopteran hosts, reflecting possible co-evolution of hosts and microsporidian isolates.

Keywords: Microsporidia; rRNA; *Nosema*; *Vairimorpha*; *Bombyx mori*

1. Introduction

Microsporidia infect a broad range of vertebrates and invertebrates including insects, fishes and mammals (Wasson and Peper, 2000; Weiss, 2001; Wittner and Weiss, 1999). They are responsible for infectious diseases in humans and considerable problems in industries such as fisheries and sericulture. Microsporidia are eukaryotes with a distinct nucleus and nuclear envelope, but they do not have centrioles or mitochondria and are considered unique among the eukaryotes in that their small subunit ribosomal RNA (SSU-rRNA) genes are smaller than those of typical eukaryotes (Vossbrinck et al., 1993). The first molecular phylogenetic analysis including a microsporidian species, *Vairimorpha necatrix*, revealed that microsporidia might have diverged from other eukaryote like forms before the symbiotic relationship developed between mitochondria and eukaryotes (Vossbrinck et al., 1987). More recent analyses using rRNA, EF 1 α and β tubulin genes support the placement of microsporidia within fungi (Fast et al., 1999; Hirt et al., 1999; Keeling and Doolittle, 1996; Keeling et al., 2000).

Of over 1200 microsporidian species described (see Wittner and Weiss, 1999), at least 200 have been assigned to the genus *Nosema* (see Sprague, 1981). This seemingly disproportionate number of *Nosema* species may be due in part to incorrect identifications. The difficulties of proper identification of a *Nosema* species, even when detailed time-course of infections and extensive light and electron microscopic examinations are carried out, are well illustrated by Mercer and Wigley (1987), who could not distinguish a *Nosema* species they had found in the poroporo stem borer, *Sceliodes cordalis*, from 12 other *Nosema* species known to infect

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Lepidoptera. In fact many of the early classifications of microsporidia based on life cycle features, spore size, shape, and ultrastructure of spores including number of coils of the polar tube and host-parasite relationship resulted in the unnecessary creation of new *Nosema* species.

Among many diseases that affect the mulberry silkworm, *Bombyx mori*, the disease caused by the microsporidian *N. bombycis* is the most devastating, since it inflicts severe silk cocoon crop loss and it is passed on to the next generation transovarially. In fact the first microsporidian identified was *Nosema bombycis* Naegeli, (1857) from silkworm. In 1865, when silk production in France plunged to a meager level because of *Nosema* infection, Louis Pasteur, who was asked to study the disease in detail, reported that obtaining spore-free mother moths is an infallible means to obtain healthy eggs (Pasteur, 1870).

In recent years, previously undescribed micrsporidia pathogenic to silkworm have been isolated from sericultural farms. Their pathogenicity, target tissues, spore structure, and transovarial transmissibility have been studied. Fujiwara (1980) reported two new isolates, NIS-M11, and NIS-M12 from the female moths of silkworm. Of these, NIS-M12 was transferred to genus *Vairimorpha* by Sato and Watanabe (1986) on the grounds of existence of two types of spores in a single cell: one type was formed through apanosporoblastic (Nosema-like) development of sporogony, while the other type of spore was formed through the pansporoblastic (Thelohania-like) pattern of development of sporogony. Subsequent studies in NIS-M11 by Iguchi et al. (1997) showed that this isolate also formed uninucleate octospores when it was raised at 18 °C prompting them to classify this isolate as *Vairimorpha* spp. Further, Fujiwara (1984) isolated *Pleistophora*—like microsporidian from the silkworm. This microsporidian invaded muscles, fat bodies, Malpighian tubules, and silkglands of the larvae. In the infected larvae, 16 or more spores are formed in a cyst. Hatakeyama et al. (1997) studied SSU-rRNA sequences of NIS-M11, NIS-M12, *Pleistophora* spp. and *N. bombycis* (strain SES-NU) and showed that NIS-M11 and NIS-M12 are closely related as reflected by 96.7% similarity in their sequences, but differed from *N. bombycis* (85.1% similarity). *Pleistophora* spp. showed the least similarity (68%) with *N. bombycis*.

In an earlier study three microsporidia were isolated from different localities of Karnataka state of India (Ananthalakshmi et al., 1994; Fujiwara et al., 1993). Fujiwara et al. (1993) identified three microsporidian isolates from the silkworm, *B. mori* based on spore shape and size and designated them as NIK-2r, NIK-3h, and NIK-4m. The spores of NIK-2r are short oval in shape and measure $3.6 \pm 0.10\,\mu m$ in length (L) by $2.8 \pm 0.02\,\mu m$ in width (W). It was isolated from the egg production center of Central Sericultural Research & Training Institute, Mysore. NIK-3h was collected from a farmer's rearing house in Hassan district of Karnataka State. Its spore size is $3.8 \pm 0.09\,\mu m$ (L) by $2.3 \pm 0.01\,\mu m$ (W) and the shape is elongate oval. NIK-4m was collected from the egg production centre of Karnataka State Sericulture Department, Mysore. Its spore size is $5.0 \pm 0.05\,\mu m$ (L) by $2.1 \pm 0.003\,\mu m$ (W) and the spore shape is long elongate oval.

As reported in the present paper, we have studied their pathogenicity, mode of transmission, tissue specificity of infection, and the small subunit rRNA (SSU-rRNA) gene sequences along with a standard isolate of *N. bombycis*. By comparing the sequences of the 16S rRNA genes of the isolates with those of other microsporidia, we have presented molecular evidence that one of the three isolates is in fact the type species while the other two isolates are *Vairimorpha* spp.

2. Materials and methods

2.1. Production and purification of microsporidian isolates

The three new microsporidian isolates and the type species, *N. bombycis* were cultured in laboratory-reared silkworm larvae at 25 °C. Soon after the fourth moult, batches of 50 healthy larvae (strain NB_4D_2) were administered per os with a spore load of 5×10^3 per larva. The spore concentrations were predetermined for each of the isolates so as to avoid early mortality. Moths derived from infected larvae were homogenated for 3 min in 0.85% NaCl followed by filtration through a cheese cloth and centrifugation at 3000*g* for 10 min. The spore pellet was Percoll-purified by gradient centrifugation as described by Canning et al., 1999a.

2.2. Examination of infected larval tissues

To identify the host tissues that were infected, different tissues (listed in Table 2) from day 3 fifth instar infected larvae (from Section 2.1) were dissected out, individually ground with a pestle and morter in physiological saline and examined for the presence of spores using phase-contrast, and Nomarski interference phase contrast microscopy as well as Giemsa staining. We did not look for tissue specificity in infected moths since many of the tissues like silk gland, gut are in atrophied state at this stage.

2.3. Pathogenicity of microsporidian isolates

Bombyx mori larvae (strain, NB_4D_2) were infected with each of the three microsporidian isolates and *N. bombycis*, to determine their pathogenicity. A control batch of larvae fed on mulberry leaves smeared with

deionised water was also maintained. For each of the three new isolates and *N. bombycis* and control, three replicates of 40 healthy larvae each were maintained. Three spore doses of three replicates of 1×10^7 (dose A), 1×10^6 (dose B) and 1×10^5 (dose C)/ml were used as infective doses on day 1 of the second instar and the actual ingested doses were estimated based on the consumed portion of the mulberry leaf coated with the infective dose. The dead larvae were checked under a phase contrast microscope every day till spinning for the presence of spores.

The pathogenicity caused by the microsporidian isolates was expressed as LT_{50} (median lethal time), the time required to cause 50 percent larval mortality from the time of infection. The comparative pathogenicity of different isolates was determined by subjecting the mortality data to Probit analysis (Finney, 1971) and the results were compared with LT_{50} (Table 3).

2.4. Mode of transmission

Fifth instar larvae (day 2) of NB_4D_2 were inoculated per os with purified spores of microsporidian isolates and *N. bombycis*. For each of the isolates, two replicates of 50 larvae each were maintained. Each replicate was given infective dose of 1×10^4 spores/ml. The inoculation doses were determined by measuring the ingested portion of the given leaf. Since we cannot assume that 100% of the larvae that ingested spores will become infected, infection levels in the larvae which were administered infective dose of spores were not known. The eggs from the infected moths derived from infected larvae were allowed to proceed to embryonic development after termination of egg diapause. Thirty eggs from each brood were crushed and examined only for microsporidian spores under student microscope at 1000× at different stages of embryonic development.

2.5. Isolation of DNA from purified spores of microsporidian isolates

Genomic DNA was extracted from the non-germinated spores of NIK-3h and germinated spores of *N. bombycis*, NIK-2r, and NIK-4m as per the procedure described by Undeen and Cockburn (1989). Briefly, the spores of NIK-3h were suspended in 100 mM NaCl, 200 mM Sucrose, 10 mM EDTA and 30 mM Tris–HCl buffer, pH 8.0. Equal volumes of spore suspension (5×10^{10}) and glass beads (0.45–0.50 mm) were pooled and shaken for 30 s at maximum speed on a cyclomixer. When >85% of the spores had ruptured, as indicated by their dark and empty appearance under a phase contrast microscope, the homogenate was used for DNA extraction.

The spores of *N. bombycis*, NIK-2r and NIK-4m were suspended separately in 100 mM NaCl, pH 9.5, Glycine–NaOH buffer (10 mM) at 30 °C for 15 min. After germination, the emptied content of the spores was used for DNA extraction.

2.6. PCR amplification and sequencing of SSU rDNA of Nosema isolates

The small subunit (SSU) rRNA gene was amplified from each of the three microsporidian isolates and *N. bombycis* using the primer 18f 5′-CACCAGGTTGATTC TGCC-3′ and 1537r 5′-TTATGATCCTGCTAATGGT TC-3′ designed by Baker et al. (1995). PCR amplification was carried out in 20 μl, using 10 ng DNA, 5 pmol of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, and 1 U of *Taq* Polymerase (MBI Fermentas, USA). The amplification conditions were: 94 °C denaturation for 3 min, followed by 30 cycles of 94 °C for 30 s, 54 °C annealing for 45 s, and extension at 72 °C for 90 s, with a final extension of 10 min on a thermal cycler (Perkin–Elmer, 9700). PCR products were directly cloned into TA cloning vector (Invitrogen). Three clones from each of the three microsporidian isolates and *N. bombycis* were sequenced using Big dye terminator chemistry on an ABI PRISM 377 automated sequencer.

2.7. Phylogenetic analysis

The species analyzed together with the accession numbers of the SSU-rRNA gene sequences are given in Table 1. Sequence homology analyses were performed using BLAST database searches. Multiple sequence alignment was performed with the GCG pileup programme (Wisconsin package, Version 10, Genome computer group). Phylogenetic tree was constructed with nucleotide sequences using molecular evolutionary genetic analysis (MEGA) (Kumar et al., 1993). Neighbor joining tree was constructed using Kimura 2-parameter with both transitions and transversions included with a bootstrap replications of 1000 and default random seed.

3. Results

3.1. Tissues infected

In fifth instar larvae, the tissue specificity of infection was examined for each of the three microsporidian isolates and *N. bombycis* (Table 2). In the fifth instar larvae infected by either NIK-2r or *N. bombycis*, the gonads were heavily infected, whereas in the NIK-4m infected larvae these tissues were devoid of infection. In NIK-3h infected larvae, infection level in the gonads was very low. Only 1 or 2 mature spores could be observed per microscopic field. The site of infection of NIK-4m was unique among the four isolates. The infected larvae

Table 1
Small subunit (SSU) ribosomoal RNA sequences used for phylogenetic analysis

Accession No	Organism name	Host	Order	Family
D85503	*N. bombycis*[1]	*B. mori*	Lepidoptera	Bombycidae
AB036052	*N. bombycis*[2]	*Antheraea Mylitta Drury*	Lepidoptera	Saturniidae
AY259631	*N. bombycis*[3]	*Helicoverpa armigera*	Lepidoptera	Noctuidae
L39111	*N. bombycis*[4]	*B. mori*	Lepidoptera	Bombycidae
AF240347	*N. bombycis*[5]	*B. mori*	Lepidoptera	Bombycidae
AY017211	***N. bombycis*[6] (NIK-2r)**	***B. mori***	**Lepidoptera**	**Bombycidae**
AY017210	***N. bombycis*[7]**	***B. mori***	**Lepidoptera**	**Bombycidae**
AY383655	*Nosema* sp.[1]	*Pieris rapae*	Lepidoptera	Pieridae
AF238239	*Nosema* sp.[2]	*Spodoptera litura*	Lepidoptera	Noctuidae
AF485270	*Nosema* sp.[3]	*P. rapae*	Lepidoptera	Pieridae
AB009977	*Nosema* sp.[4]	*Antheraea mylitta*	Lepidoptera	Saturniidae
AF141130	*Nosema* sp.[5]	*Noctuid moth*	Lepidoptera	Noctuidae
AY017212	**NIK-3h**	***B. mori***	**Lepidoptera**	**Bombycidae**
AY017213	**NIK-4m**	***B. mori***	**Lepidoptera**	**Bombycidae**
AF240348	*Nosema* sp. isolate 1	*Calospilos suspecta*	Lepidoptera	Geometridae
AF240349	*Nosema* sp. isolate 2	*B. mori*	Lepidoptera	Bombycidae
AF240350	*Nosema* sp. isolate 3	*B. mori*	Lepidoptera	Bombycidae
AF240351	*Nosema* sp. isolate 4	*B. mori*	Lepidoptera	Bombycidae
AF240353	*Nosema* sp. isolate 6	*Phyllobrotica armata Baly*	Coleoptera	Chrysomelidae
AF240354	*Nosema* sp. isolate 7	*P. rapae*	Lepidoptera	Pieridae
D85502	*Vairimorpha* sp.[1]	*B. mori*	Lepidoptera	Bombycidae
D85501	*Vairimorpha* sp.[2]	*B. mori*	Lepidoptera	Bombycidae
L39114	*Vairimorpha* sp.[3]	*B. mori*	Lepidoptera	Bombycidae
AJ131645	*V. imperfecta* isolate 1	*Plutella xylostella*	Lepidoptera	Plutellidae
AJ131646	*V. imperfecta* isolate 2	*P. xylostella*	Lepidoptera	Plutellidae
U09282	*Nosema trichoplusiae*	*Trichoplusia ni*	Lepidoptera	Noctuidae
AJ012606	*Nosema tyriae*	*Tyria jacobaeae*	Lepidoptera	Arctiidae
AJ011833	*Nosema granulosis*	*Gammarus duebeni*	Amphipoda	Gammaridae
U26532	*Nosema furnacalis*	*Ostrinia furnacalis*	Lepidoptera	Pyralidae
AF327408	*Vairimorpha cheracis*	*Cherax destructor destructor*	Decapoda	Parastacidae
U26534	*Nosema apis*	*Apis mellifera*	Hymenoptera	Apidae
Y00266	*V. necatrix*	*Pseudaletia unipuncta*	Lepidoptera	Noctuidae
AY008373	*Nosema bombi*	*Bombus terrestris*	Hymenoptera	Apidae
AF426104	*N. carpocapsae*	*Cydia pomonella*	Lepidoptera	Tortricidae
U27359	*Nosema oulemae*	*Oulema melanopus*	Coleoptera	Chrysomelidae
U26533	*Nosema ceranae*	*Apis cerana*	Hymenoptera	Apidae
AF033315	*Vairimorpha lymantriae*[1]	*Lymantria dispar*	Lepidoptera	Lymantriidae
AF141129	*V. lymantriae*[2]	*L. dispar*	Lepidoptera	Lymantriidae
AF033316	*N. Portugal*	*L. dispar*	Lepidoptera	Lymantriidae
AF177920	*Encephalitozoon hellem*[1]	*Agapornis roseicollis*	Psittaciformes	Psittacidae
L39108	*E. hellem*[2]	*Homo sapiens*	Primates	Hominidae
L17072	*Encephalitozoon cuniculi*[1]	*H. sapiens*	Primates	Hominidae
L39107	*E. cuniculi*[2]	*H. sapiens*	Primates	Hominidae
L39113	*Septata intestinalis*[1]	*Homo sapiens-intestine*	Primates	Hominidae
L19567	*S. intestinalis*[2]	*H. sapiens-intestine*	Primates	Hominidae
L16868	*Enterocytozoon bieneusi*	*H. sapiens*	Primates	Hominidae
U10883	*Enterocytozoon salmonis*	*Oncorhynchus tshawytscha*	Salmoniformes	Salmonidae
U11046	*Vittaforma corneae*[1]	*Homo sapiens-eye*	Primates	Hominidae
L39112	*V. corneae*[2]	*H. sapiens-eye*	Primates	Hominidae
D85500	*Pleistophora* sp.[1]	*B. mori*	Lepidoptera	Bombycidae
AY009115	*Endoreticulatus bombycis*	*B. mori*	Lepidoptera	Bombycidae
L39109	*Endoreticulatus schubergi*	*H. sapiens*	Primates	Hominidae
AF025685	*Amblyspora connecticus*	*Aedes salinarius*	Diptera	Culicidae
AF027683	*C. lunata*	*Culex pilosus*	Diptera	Culicidae

The three new isolates and the *N. bombycis* strain used in the present study are indicated in bold.

carried sacs of hypertrophied infected midgut tissues (Fig. 1A). These sacs harbored mature spores (Fig. 1B) suggesting that the high rate of proliferation was responsible for massive and rapid destruction of midgut tissues.

These observations show that the three microsporidian isolates could be categorised into: NIK-2r which, similar to *N. bombycis*, invaded all the tissues examined; NIK-3h which invaded multiple tissues, but lacked discernible infection of the midgut epithelium,

Table 2
Sites of infection of host tissues by the new microsporidian isolates and *N. bombycis*

Host tissue	*N. bombycis*	New microsporidian isolates		
		NIK-2r	NIK-3h	NIK-4m
Fat body	++	++	+	–
Gonads	++	++	+	–
Gut epithelium	++	++	–	+++
Gut muscle	++	++	+	–
Malpighian	++	++	++	–
Tubules	++	++	++	–
Muscles	++	++	+	–
Tracheal	++	++	++	–
Epithelium	++	++	+	–
Silk glands	++	++	+	–
Integument				
Gangila				

+++, very high infection; ++, high infection; +, low infection; –, no infection.

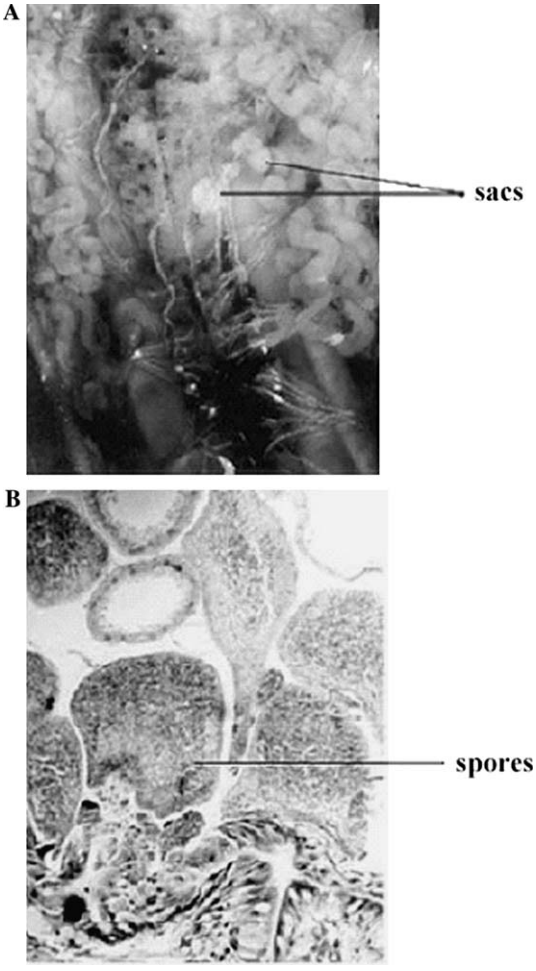


Fig. 1. (A) Hypertrophied sacs of the host midgut infected with NIK-4m. (B) Transverse section of the sacs. Scale bar is 1 mm.

and NIK-4m which infected only midgut tissue and spores accumulated in hypertrophied sacs of midgut tissues.

3.2. Pathogenicity

Pathogenicity of the three isolates and *N. bombycis* is given in Table 3. As could be seen from the mortality values, the rate of mortality was dose dependent in all the cases but differed between different isolates.

The isolate NIK-4m was found to be the most virulent as compared to the other isolates. At all the three infective doses, some 3rd instar larvae succumbed to infection while the other three did not result in any mortality at this stage. The highest dose (10^7) of NIK-4m resulted in the mortality of all the larvae by the end of 4th instar (Table 3). The mortality was 100% by the time larvae reached 5th instar in NIK-2r and *N. bombycis* at all the three doses whereas in case of NIK-3h some larvae infected with the lowest dose survived and pupated but subsequently succumbed to infection at all the three doses

Among the three new isolates and *N. bombycis*, NIK-4m was the most virulent resulting in 50 percent mortality by 10.70 days pi at the dose of 10^7 followed by NIK-2r, *N. bombycis* and NIK-3h with the corresponding LT_{50} values of 13.27, 13.55, and 17.40 days pi. At the spore concentrations of 10^6 and 10^5 also, NIK-4m was the most virulent followed by NIK-2r, *N. bombycis* and NIK-3h (Table 3). The overall pathogenicity of the isolates based on LT_{50} values was in the order of NIK-4m > NIK-2r = *N. bombycis* > NIK-3h.

3.3. Mode of transmission

To check for transovarial transmission, the eggs laid by the infected mother moths were examined. The results of incidence of infection of different microsporidians during embryonic development are plotted in Fig. 2. As could be seen, only NIK-2r and *N. bombycis* transmitted infection transovarially to the embryos whereas the eggs laid by NIK-3h and NIK- 4m infected moths were devoid of any infection suggesting that these two isolates are not transovarially transmitted. On day 4 of the embryonic development only 61 spores were observed in embryos derived from *N. bombycis* and NIK-2r infected larvae, which increased to 768 spores and 940 spores respectively, at day 10 of the embryonic development. There was a sharp increase in spore production of day 8 to day 10 of embryonic development. These results indicate that the vegetative cells of microsporidia were maturing into spores as the embryonic development progressed over time.

3.4. Molecular phylogeny

We compared the SSU-rRNA gene sequence from the three isolates and *N. bombycis* with the corresponding rRNA gene regions in other microsporidia (Table 1). The sequences of *Amblyospora connecticus* and *Culicosporella lunata* were used as outgroups. Phylogenetic

Table 3
Comparative pathogenicity of new microsporidian isolates and *N. bombycis* (agewise mortality)

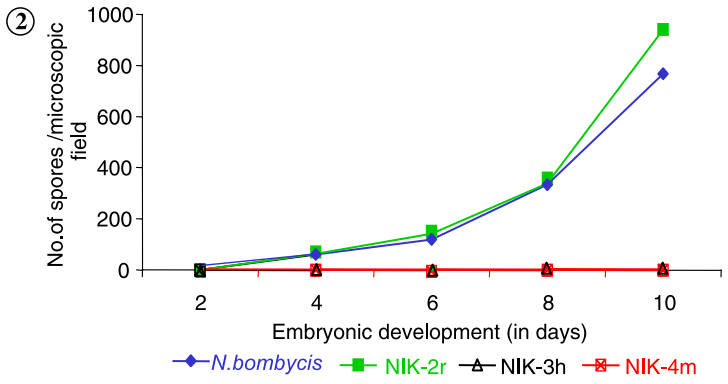
Microsporidian isolates	Inoculation ingested (dose/ml) (dose/larva)	Larval mortality (instarwise)								Pupae				Median lethal time (LT_{50}) (days)	Fiducial limits (days)	Regression equation	Error (b)
		3rd		4th		5th		Before spinning		Survival		Dead					
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%				
N. bombycis	A 10^7 (3.2×10^4)	—	—	110	91.7	10	8.3	—	—	—	—	—	—	13.55	13.35–13.74	$Y = -12.5761 + 15.5294X$	0.7698
	B 10^6 (3.3×10^3)	—	—	99	82.5	21	17.5	—	—	—	—	—	—	14.42	14.24–14.59	$Y = -17.7711 + 19.6497X$	1.0307
	C 10^5 (3.7×10^2)	—	—	49	40.8	71	59.2	—	—	—	—	—	—	15.83	16.02–16.21	$Y = -16.6441 + 17.5452X$	0.9547
NIK-2r	A 10^7 (2.0×10^4)	—	—	103	85.8	17	14.2	—	—	—	—	—	—	13.27	13.08–13.45	$Y = -13.3063 + 16.3046X$	0.8078
	B 10^6 (3.2×10^3)	—	—	96	80	24	20	—	—	—	—	—	—	14.82	14.62–15.01	$Y = -13.9732 + 16.2065X$	0.7856
	C 10^5 (3.4×10^2)	—	—	60	50	60	50	—	—	—	—	—	—	15.64	15.44–15.83	$Y = -16.3947 + 17.9163X$	0.9316
NIK-3h	A 10^7 (3.9×10^4)	—	—	26	21.7	72	60	22	18.3	—	—	—	—	17.40	17.20–17.60	$Y = -19.1161 + 19.4397X$	0.9899
	B 10^6 (3.6×10^3)	—	—	13	10.8	69	57.5	38	31.7	—	—	—	—	18.00	17.79–18.21	$Y = -18.9277 + 19.0626X$	1.0208
	C 10^5 (3.9×10^2)	—	—	8	6.6	47	39.2	41	34.2	12	10	12	10	18.71	18.46–18.96	$Y = -15.3452 + 15.9931X$	1.1150
NIK-4m	A 10^7 (2.0×10^4)	42	35	78	65	—	—	—	—	—	—	—	—	10.7	10.61–10.78	$Y = -3.5710 + 8.3274X$	0.4229
	B 10^6 (3.9×10^3)	18	15	64	53.4	28	23.3	9	7.5	1	0.8	—	—	13.32	13.23–13.41	$Y = -5.0185 + 8.9094X$	0.4456
	C 10^5 (3.3×10^2)	7	5.8	70	58.4	32	26.7	10	8.3	1	0.8	—	—	13.92	13.83–14.00	$Y = -6.5482 + 10.0991X$	0.5299

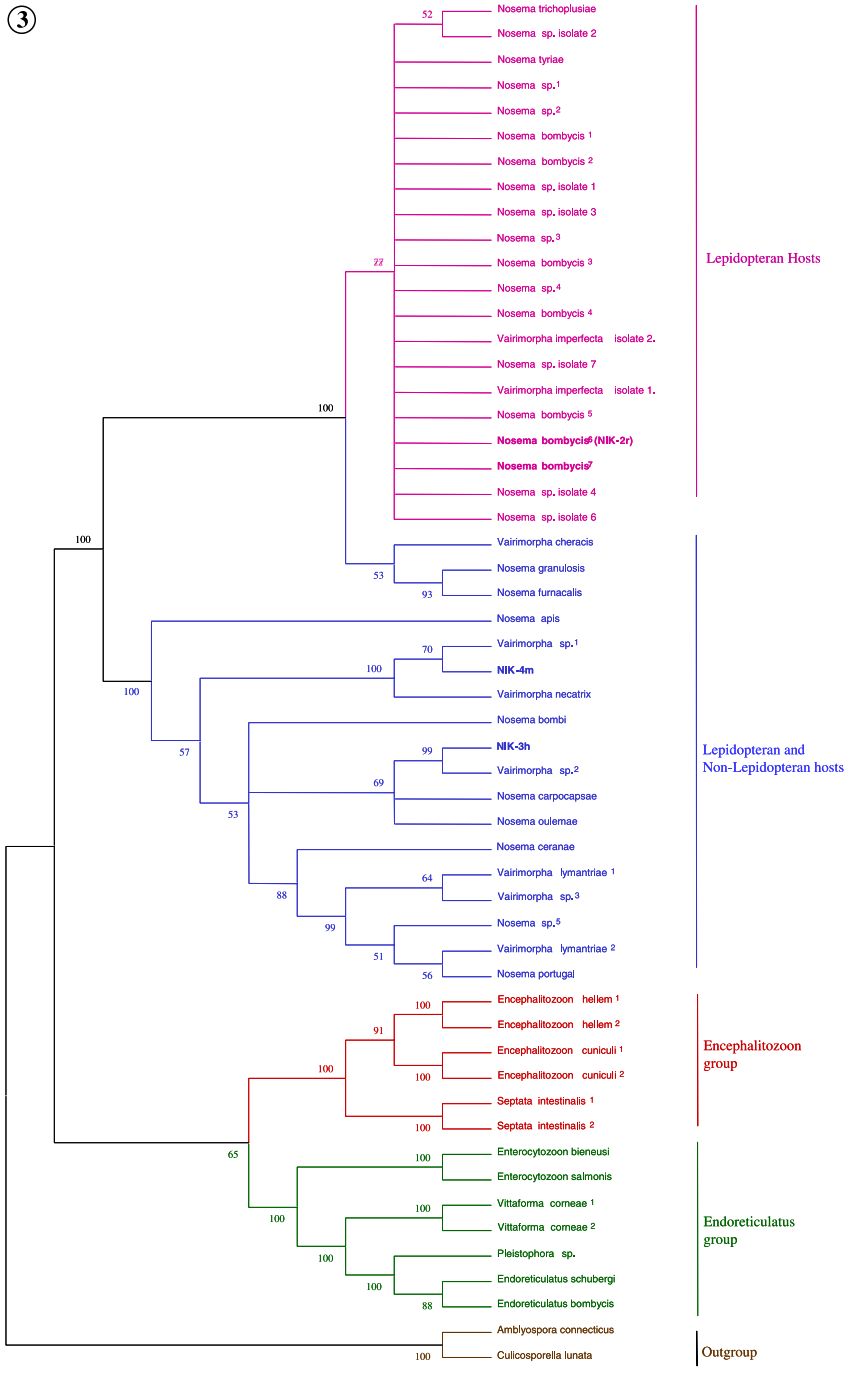
analysis of the aligned sequences was carried out by using MEGA programme and the phylogenetic tree generated is shown in Fig. 3. With the exception of *Vairimorpha imperfecta*, all the *Nosema* species which parasitise lepidopterans formed a distinct clade whereas all the species of *Vairimorpha* got clustered along with the *Nosema* species of non-lepidopteran insects and three lepidopteran *Nosema* species (*Nosema* sp[5], *Nosema Portugal* and *Nosema carpocapsae*). The separation of the two clades was quite robust as evidenced by 100 percent bootstrap values. We compared SSU-rRNA sequence of NIK-2r with corresponding sequences of several strains of *N. bombycis* (Table 1). Altogether we observed nine nucleotide differences among the strains studied and the pair-wise comparison showed that the strains differed from each other by 4–6 bases and showed >99% similarity among themselves. Several complete sequences of 16S rRNA sequences attributed to *N. bombycis* have been deposited in GenBank. In order to avoid the problems associated with the sequence errors in the unpublished sequences accessible through GenBank, we included only sequences published in the peer-reviewed journals with the exception of Accession No. D85503, the sequence from the stock of *N. bombycis* (SES-NU) isolated from *B. mori* held at the National Sericultural Experimental Station, Tokyo. Canning et al., 1999b suggested that the determination of the species status of other isolates of *Nosema* should be made against D85503. NIK-2r differed by 5 bases from *N. bombycis* used in the present study and 6 bases from D85503, which account for ~0.5% of the total sequence difference. Although it is not known about the number of nucleotide differences, which represent intraspecific variation in *N. bombycis*, we believe that the difference of nine bases falls within intraspecific variation. The tree places NIK-2r along with all the *N. bombycis* strains and most of the *Nosema* species of lepidopteran origin with a bootstrap value of 77%.

NIK-3h and NIK-4m isolates were grouped in a clade that included *V. necatrix* and most of the *Nosema* species of non-lepidoterans with a bootsrap value of 100%. NIK-4m showed clear nucleotide similarity to *V. necatrix* (98.8%), the type species of *Vairimorpha* and

Fig. 2. Infection level during embryonic development of NB_4D_2 transovarially infected with new microsporidian isolates and *N. bombycis*.

Fig. 3. The phylogenetic relationship of the three new isolates, NIK-3h, NIK-4m, and NIK-2r as determined by 16S SSU-rRNA sequence analysis using MEGA programme. Neighbor joining tree was constructed using Kimura 2-parameter. Numbers on the nodes indicate the number of times a particular branch was recorded per 100 bootstrap replications following 1000 replicates. The branches with lower than 50% confidence values were ignored. The four isolates used in the present study are indicated in bold.


②
No.of spores /microscopic field
1000
800
600
400
200
0
2
4
6
8
10
Embryonic development (in days)
N.bombycis
NIK-2r
NIK-3h
NIK-4m


③
Nosema trichoplusiae
Nosema sp. isolate 2
Nosema tyriae
Nosema sp.1
Nosema sp.2
Nosema bombycis 1
Nosema bombycis 2
Nosema sp. isolate 1
Nosema sp. isolate 3
Nosema sp.3
Nosema bombycis 3
Nosema sp.4
Nosema bombycis 4
Vairimorpha imperfecta isolate 2.
Nosema sp. isolate 7
Vairimorpha imperfecta isolate 1.
Nosema bombycis 5
Nosema bombycis6 (NIK-2r)
Nosema bombycis7
Nosema sp. isolate 4
Nosema sp. isolate 6
Vairimorpha cheracis
Nosema granulosis
Nosema furnacalis
Nosema apis
Vairimorpha sp.1
NIK-4m
Vairimorpha necatrix
Nosema bombi
NIK-3h
Vairimorpha sp.2
Nosema carpocapsae
Nosema oulemae
Nosema ceranae
Vairimorpha lymantriae 1
Vairimorpha sp.3
Nosema sp.5
Vairimorpha lymantriae 2
Nosema portugal
Encephalitozoon hellem 1
Encephalitozoon hellem 2
Encephalitozoon cuniculi 1
Encephalitozoon cuniculi 2
Septata intestinalis 1
Septata intestinalis 2
Enterocytozoon bieneusi
Enterocytozoon salmonis
Vittaforma corneae 1
Vittaforma corneae 2
Pleistophora sp.
Endoreticulatus schubergi
Endoreticulatus bombycis
Amblyospora connecticus
Culicosporella lunata
Lepidopteran Hosts
Lepidopteran and Non-Lepidopteran hosts
Encephalitozoon group
Endoreticulatus group
Outgroup

NIK-3h showed closer relatedness to *Vairimorpha* spp. (NIS-M11) (99.2%) isolated from *B. mori.*

4. Discussion

4.1. Tissue specificity

Gonadal infection with *Nosema* spp. has an important bearing on the vertical transmission of the parasite through transovarial transmission. This aspect is important in sericulture industry since silkworm eggs distributed to farmers are required to be certified 'microsporidian free.' In the present study NIK-2r and *N. bombycis* infected all of the tissues examined including midgut and gonads. On the other hand, of the other two isolates, NIK-4m infected heavily only midgut epithelium and showed no infection of the gonads and other tissues examined whereas NIK-3h did not show any discernible infection of the midgut and very mild to high infection of the gonads and other tissues.

The pathogenicity of the isolates used in the present study seems to be proportional to the level of infection of the midgut. NIK-4m, which heavily infected the midgut epithelium, was the most pathogenic while NIK-3h, which did not show apparent infection of the midgut, was the least pathogenic. The NIK-4m infected midgut tissues were seen as greatly hypertrophied bags ofspores. *N. bombycis* and NIK-2r which infect ovary, are transmitted transovarially to the next generation. The lack of transovarial transmission and high degree of pathogenicity in NIK-4m suggest that the pathogen possibly ensures its perpetuation through efficient horizontal transmission. Our results on lack of transovarial transmission of NIK-3h are not surprising considering that we noticed very low level of infection of ovary of the infected larvae. However, we cannot exclude the possibility that transovarial transmission occurs although we were unable to detect it. The low level of infection of NIK-3h of the gonadal tissues was seen in the ''tissue specificity'' experiments since larvae were inoculated soon after fourth moult. Perhaps inoculation of earlier instars with NIK-3h and examination of the offspring derived from the infected adults would clarify the transovarial transmission status of this isolate.

4.2. Phylogenetic status of the new microsporidian isolates

Development of molecular tools, in particular, comparative studies of rRNA genes have been shown to be very useful in microsporidian taxonomy and phylogeny (Baker et al., 1994; Canning et al., 1999a,b; Malone and McIvor, 1996.). We observed more than 99.5% nucleotide similarity between the new isolate NIK-2r and *N. bombycis* used in the present study and other *N. bombycis* strains whose sequences are available in the GenBank. Besides, many life cycle features of NIK-2r showed very close similarity to type species, *N. bombycis*. Iwano and Ishihara (1991) showed that *N. bombycis* produces early spores responsible for within host transmission with only 3–5 coils of the polar tube and that spores produced later in infection, which have 11 or more coils of polar tube are responsible for between host transmission. In the present study in both NIK-2r and *N. bombycis* early spores which were pyriform in shape appeared 72 hpi and spore with 11–13 coils appeared after 96 hpi (data not shown). Both NIK-2r and *N. bombycis* transmitted the infection transovarially to the progeny eggs at a frequency of 100% and infection was observed in most of the larval tissues as reported earlier by Fujiwara, 1979; Han and Watanabe, 1988. All these results amply suggest that NIK-2r is in fact *N. bombycis*, and not a new microsporidian species.

The nucleotide differences in the SSU-rRNA gene, the site of infection, pathogenicity, and mode of transmission clearly delineated the two isolates, NIK-4m and NIK-3h from the type species, *N. bombycis* (Fig. 3). The SSUrRN gene sequence data, high pathogenicity, and formation of hypertrophied sacs of midgut tissues suggest a close similarity of NIK-4m to *V. necatrix* than to the lepidopteran—infecting *Nosema* species. The preliminary observations suggest that NIK-4m has features such as diplokaryotic free spores and formation of octospores, which are common features of the genus, *Vairimorpha*. The SSU-rRNA sequence of NIK-3h, on the other hand, group this isolate in the clade containing *Vairimorpha* sp. (NIS-M11) reported by Fujiwara (1980), which produces eight uninucleate octospores in the silkworm hosts raised at 18 °C (Iguchi et al., 1997). Taken together, these results suggest that NIK-4m and NIK-3h isolates represent two new species of silkworm microsporidia that belong to the genus *Vairimorpha*. Further studies on life cycle and ultrastructure features of the two isolates will strengthen their taxonomic status which we have based on the SSU-rRNA sequence data.

The intraspecific nucleotide diversity, based on the published sequences of *N. bombycis* isolates, suggests that only differences of 4–6 bases that account for ~0.5% of the total sequence exist among the isolates. We suggest that the taxonomic status of any new *N. bombycis* isolates may be determined by using *N. bombycis* sequence reported in the present study (AY017210) or D85503 as suggested by Canning et al., 1999b.

In the phylogenetic tree outlined here, all *Nosema* species and a lone species, *V.imperfecta*, all of which infect Lepidoptera with the exception of *Nosema* species isolate six isolated from a beetle, *Phyllobrotica armatabaly*, belong to one clade. On the other hand, almost all the *Vairimorpha* spp. and *Nosema* spp. which infect Hymenoptera, Coleoptera and Lepidoptera came out in an another branch. These results clearly suggest that

Nosema species which infect only Lepidoptera species are more closely related to each other than they are to the other non-lepidopteran *Nosema* species and seem to have phylogenetic significance as opined by Baker et al. (1994). The distinct grouping of *Nosema* species which parasitise only Lepidoptera suggests the probable co-evolution of lepidopteran *Nosema* species with their host group over a period of time.

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