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ORIGINAL PAPER

Morphological and phylogenetic analysis of *Nosema* sp. HR (Microsporidia, Nosematidae): a new microsporidian pathogen of *Histia rhodope* Cramer (Lepidoptera, Zygaenidae)

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Abstract A new microsporidium was isolated from *Histia rhodope* Cramer (Lepidoptera, Zygaenidae), a pest of *Bischofia javanica* BL. in China. The morphology and molecular systematic of this novel microsporidian isolate had been described in this study. The spores were long oval and measured 3.1×1.9 μm on fresh smears. Ultrastructure of the spores was characteristic for the genus *Nosema*: 14–15 polar filament coils, posterior vacuole, and a diplokaryon. The sequenced *rRNA* gene of this isolate is 4309 bp long. The organization of the *rRNA* gene is 5′-LSU rRNA-ITS-SSU rRNA-IGS-5S-3′, which is similar to that of other *Nosema* species (such as *Nosema bombycis*). Phylogenetic analysis based on *LSU rRNA* gene and *SSU rRNA* gene both revealed that this novel micorsporidian which isolated from *H. rhodope* had close relationship to the genus *Nosema*. Additionally, this isolate can also cause systemic infection of *Bombyx mori*. So, we should pay attention not only to *N. bombycis*, but also to other microsporidian (such as *Nosema* sp. HR) in sericulture in the future.

Keywords Microsporidian · *Nosema sp.* HR · rRNA · Morphology · Phylogenetic analysis

Nucleotide sequence reported in this study has been submitted to the GenBank™, EMBL, and DDBJ databases under the accession number KP100640.

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Introduction

Microsporidia are eukaryotic organisms that parasitize nearly all groups of animals, ranging from protists to invertebrates and vertebrates, including human beings (Franzen and Muller 1999; Joseph et al. 2006). These organisms are serious pests in sericulture, apiculture, and fisheries (Wittner and Weiss 1999; Abdel-Ghaffar et al. 2012). Of the 1300 microsporidian species described in the literatures so far (Corradi et al. 2009), at least 200 belong to the genus *Nosema* (Sprague 1982). Additionally, the most general microsporidian that infect Lepidoptera are members of the genus *Nosema* and *Vairimorpha*. And, the genera *Nosema* and *Vairimorpha* could not be separated into different clades using molecular characteristics (Tsai et al. 2003; Ku et al. 2007).

In 2014, a novel microsporidian was isolated from *Histia rhodope* Cramer (Lepidoptera, Zygaenidae), a pest of *Bischofia javanica* BL. in Chongqing, China. We reared this isolate in the laboratory and try to infect the domesticated silkworm *Bombyx mori* using this microsporidian. Like *Nosema bombycis* and *Vairimorpha* sp. BM (Liu et al. 2012), this new microsporidian also can cause systemic infection of *B. mori*. This isolate appeared to be morphologically different to that of the *N. bombycis*. A preliminary study revealed that the small subunit (SSU) rRNA and large subunit (LSU) rRNA of this isolate have high similarity to those of *Nosema spodopterae*. The present study described this new isolate (designated as *Nosema* sp. HR) based on its morphological characteristic and phylogenetic relationship with other micorsporidian.

Materials and methods

Host insect and microsporidian isolate

Inseminated female *H. rhodope* Cramer (Lepidoptera, Zygaenidae) moths which were infected by microsporidian

were collected from *Bischofia polycarpa* in Chongqing, China. The moths were allowed to oviposit, and the larvae were reared on leaves of *B. plycarpa* in the laboratory at room temperatures. The fifth instar larvae was dissected and examined by microscopy. Larvae with microsporidiosis were stored at 4 °C.

Spore production and purification

Nosema sp. HR was isolated from infected *H. rhodope* Cramer. The infected larvae were dissected, homogenized, and centrifuged to purify mature microsporidian spores preliminarily as described (Liu et al. 2008). Spores were further purified by discontinuous Percoll density gradient (25, 50, 75, and 100 %, *v*/*v*) and centrifuged at 30,000*g* for 40 min as described previously (Liu et al. 2008, 2013). The pellets of mature spores were rinsed several times and stored as pellets at 4 °C. The purified spores (n=50) were measured under a light microscope (OLYMPUS BX51 TRF) with an ocular micrometer and photographed with the Microscope USB Camera (OLYMPUS DP71).

Transmission electron microscopy

Electron microscopy was performed as previously described (Choi et al. 2002) with slight modifications. The purified spores of *Nosema* sp. HR were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight. Samples were postfixed for 3 h in 1 % osmium tetroxide, dehydrated through ascending ethanol series, and embedded in Epon-Araldite. Ultrathin sections were cut by using the Reichert-Jung ULTRACUT E ultramicrotome and stained in methanolic uranyl acetate, then in lead citrate. The stained grids were rinsed six times in ddH_2O, dried, examined, and photographed with a HITACHI H-7500 TEM transmission electron microscope at an accelerating voltage of 80 kV.

Genome DNA extraction and rDNA amplification

Genomic DNA was extracted as previously described (Liu et al. 2008). The primer sets used for *rRNA* gene amplification and the expected sized of all amplicons are shown in Table 1. All primers are from the report of Huang et al. (2004). The amplification was performed under the following conditions: After initial denaturation of DNA at 94 °C for 5 min, 30 cycles were run at 94 °C for 1 min, annealing temperatures for 1 min, and 72 °C for 2 min with a 10-min 72 °C extension. The polymerase chain reaction products of expected size were purified using DNA Extraction and Purification Kit and cloned into pMD19-T Vector and sequenced by Invitrogen Company.

Molecular phylogenetic analysis

Using the sequenced rDNA sequence of *Nosema* sp. HR and other sequences which were obtained from the NCBI GenBank database (Table 2), all these sequences were aligned by the ClustalX 1.83 program. The rDNA sequence of *Encephalitozoon cuniculi* was used as outgroup. Phylogenetic trees were reconstructed using the neighbor-joining method (Saito and Nei 1987) implemented in MEGA 4.0 program (Tamura et al. 2007). Bootstrap support was evaluated based on 1000 replicates.

Results

Morphological characteristics

H. rhodope Cramer (Lepidoptera, Zygaenidae) is a pest of tree *B. polycarpa*. The four stages of this pest are the eggs, larvae, pupa, and adults (moths) (Fig. 1a–d). Every stages of

Table 1 Primers used for amplification of *Nosema* sp. HR rRNA

Primer	Sequence	Amplicon size (bp)
Small subunit (SSU) rRNA		1233
18f	5′-CACCAGGTTGATTCTGCC-3′	
1537r	5′-TTATGATCCTGCTAATGGTTC-3′	
Large subunit (LSU) rRNA		2375
LSUF	5′-ACTCTCCTCTTTGCCTCAATCA-3′	
ILSUR	5′-ACCTGTCTCACGACGGTCTAAAC-3′	
Internal Transcribed spacer (ITS)		506
ILSUF	5′-TGGGTTTAGACCGTCGTGAG-3′	
S33R	5′-ATAGCGTCTACGTCAGGCAG-3′	
Intergenic spacer (IGS)		854
HG4F	5′-GCGGCTTAATTTGACTCAAC-3′	
5SR	5′-TACAGCACCCAACGTTCCCAAG-3′	

Table 2 NCBI GenBank accession numbers for of *Nosema* sp. HR and other species isolated from insects used to construct the rDNA phylogenetic tree

Microsporidia	Host	GenBank Accession No.
Encephalitozoon cuniculi	Mammal	L39107
Nosema antheraeae	*Antheraea pernyi*	EU864526
Nosema spodopterae	*Spodoptera litura*	AY211392
Nosema bombycis	*Bombyx mori*	EU350392
Nosema furnacalis	*Ostrinia furnacalis*	U26532
Nosema apis	*Apis mellifera*	U26534
Oligosporidium occidentalis	*Metaseiulus occidentalis*	AF495379
Nosema bombi	*Bombus spp.*	AY008373
Thelohania disparis	*Lymantria dispar*	DQ272237
Nosema ceranae	*Apis cerana*	FJ481912
Vairimorpha sp.	*Agrilus anxius*	GQ379702
Vairimorpha lymantriae	*Lymantria dispar*	AF033315
Nosema portugal	*Lymantria dispar*	AF033316
Nosema vespula	*Helicoverpa armigera*	U11047
Nosema oulemae	*Oulema melanopus*	U27359
Nosema thomsoni	*Choristoneura conflictana*	EU219086
Nosema carpocapsae	*Cydia pomonella*	AF426104
Vairimorpha sp.	*Bombyx mori*	D85502
Nosema necatrix	*Apis cerana*	U11051
Vairimorpha ceraces	*Cerace stipatana*	EU267796
Vairimorpha sp. BM	*Bombyx mori*	HQ891818
Vairimorpha necatrix	*Pseudaletia unipuncta*	DQ996241
Vairimorpha necatrix	*Pseudaletia unipuncta*	EU544672
Vairimorpha necatrix	*Pseudaletia unipuncta*	DQ996242
Nosema sp. C01	*Pieris rapae*	AY383655
Nosema clone MPr	*Pieris rapae*	HQ399665
Microsporidium 57864	Lepidopteran	U90885
Nosema bombi	*Bombus spp.*	AY741111
Nosema ceranae	*Apis cerana*	DQ486027
Nosema apis	*Apis mellifera*	U97150
Nosema granulosis	*Gammarus duebeni*	DQ996239
Vairimorpha cheracis	*Cherax destructor*	DQ996240
Nosema sp. SC	*Philosamia cynthia ricini*	FJ767862
Nosema bombycis	*Bombyx mori*	AY211393
Nosema spodopterae	*Spodoptera litura*	AY747307
Nosema heliothidis	*Helicoverpa armigera*	FJ772435
Nosema fumiferanae	*Choristoneura fumiferana*	HQ457432
Nosema trichoplusiae	*Trichoplusia ni*	DQ996243
Nosema plutellae	*Plutella xylostea*	AY960987
Nosema empoascae	*Empoasca fabae*	DQ996237
Nosema sp. HR	*Histia rhodope*	KP100640

H. rhodope Cramer can be infected by *Nosema* sp. HR spores. Light microscopy revealed that fresh *Nosema* sp. HR spores were generally long oval. They had a mean length and mean width of 3.1 μm (SD 0.2) and 1.9 μm (SD 0.3), respectively (Fig. 1e). Electron micrography of a longitudinal section of a mature spore revealed that the spore wall consisted of an electron-dense exospores (thickness approximately 40 nm) and electron-lucent endospore layer and that the sporoplasm was enclosed by a plasma membrane. The coiled region of the polar tube comprised 14–15 turns, and the diplokaryotic nuclei were slightly separated from each other. A membrane-bound vacuole with amorphous content was located at the posterior end of the spore (Fig. 1f). All the above-mentioned features correspond to the principle characteristics of the genus *Nosema*. Using the same method of feeding the spores of *N. bombycis* to silkworm (Liu et al. 2008), the spores of *Nosema* sp. HR had been fed to silkworm *B. mori* (Dazao). There are dead larvae in the infected silkworm after 13 days of feeding. The dead larvae were dissected and examined by light microscopy. We found that this microsporidian can cause systemic infection. The spores have been detected in the alimentary canal, silk glands, fat bodies, malpighian tubules, muscle, and gonads, etc.

Analysis of the rRNA sequence of *Nosema* sp. HR

Using four pairs of primers, we amplified the *LSU rRNA*, *SSU rRNA*, *ITS rRNA*, *IGS rRNA*, and *5S rRNA* genes, respectively. The complete sequence of the *Nosema* sp. HR *rRNA* gene is 4309 bp long (GenBank Accession No. is KP100640). The gene arrangement from the 5′ end is as follows: the large-subunit gene (*LSU rRNA*, 2497 bp), internal transcribed spacer (*ITS*, 184 bp), small-subunit gene (*SSU rRNA*, 1233 bp), intergenic spacer (*IGS*, 280 bp), and *5S rRNA* gene (*5S rRNA*, 115 bp). The sequence identity of the *Nosema* sp. HR *rRNA* to the *rRNA* of other sequenced microsporidian species has been compared. The *LSU rRNA* and *SSU rRNA* regions of *Nosema* sp. HR *rRNA* show 96–98 % identity with the corresponding regions in the *rRNA* of Nosema species (*N. spodopterae*, *Nosema* sp. SC, *Nosema* sp. HA, *N. bombycis*, *Nosema disstriae* isolate 04-14, *Nosema* sp. PX1, and *Nosema heliothidis*). The results also suggest that this isolate may be most closely related to *N. spodopterae*, with which it shares 98 % identity of total *rRNA* gene (LSU, 99 %; ITS, 89 %; SSU, 99 %; 5S, 98 %). The *rRNA* of the novel isolate also shares high sequence similarity (96 % identity) with that of the *Vairimorpha cheracis* (GenBank Accession No. DQ996240). Also, the nucleotide difference of rDNA units between *Nosema* sp. HR and other microsporidian (*N. spodopterae*, *N. bombycis*, *Nosema fumiferanae*, and Microsporidium 57864) has been counted (Table 3). According to this table, we can find that *Nosema* sp. HR has a few nucleotide differences to the "true" Nosema species on LSU, SSU, and 5S rDNA sequences. But, many nucleotide differences (more than 200 on LSU and SSU rDNA) have been detected on microsporidian which has different rDNA organization to *Nosema* sp. HR.

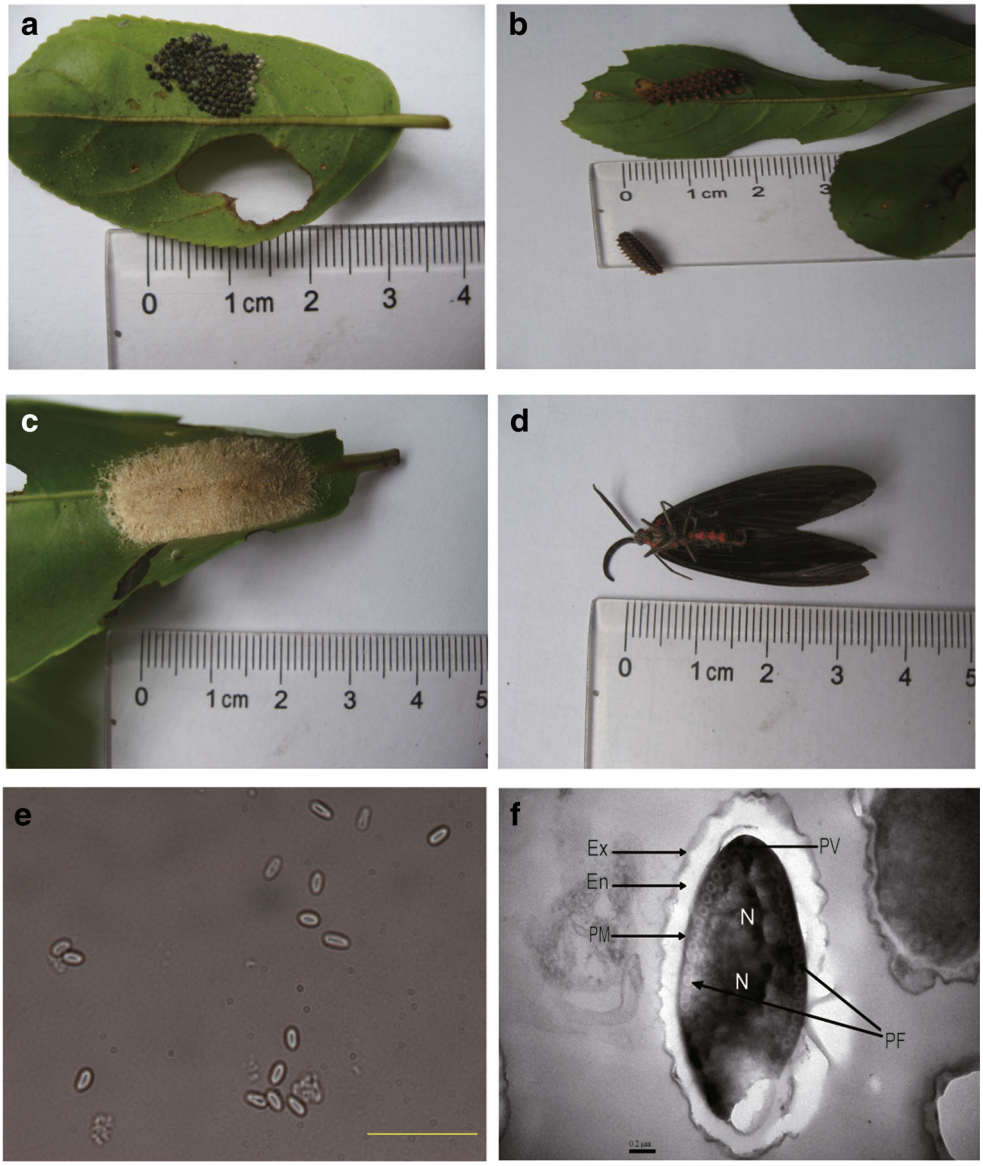

Fig. 1 Photographs of *Nosema* sp. HR spores and its host insect *Histia rhodope* Cramer. (**a**–**d**). Photograps of the eggs, larvae, cocoon, and moth of *Histia rhodope* Cramer, respectively. **e** Light micrograph of the *Nosema* sp. HR spores after Percoll purification. *Scale bar*=10 μm. **f** Electron micrograph of a longitudinal section of a *Nosema* sp. HR spore. The nucleus (*N*), exospore (*Ex*), endospore (*En*), plasma membrane (*PM*), polar filament (*PF*), and posterior vacuole (*PV*) are visible. *Scale bar*=0.2 μm

Molecular phylogeny

The *LSU rRNA* gene of *Nosema* sp. HR consists of 2497-bp nucleotides, and the GC content is 31.84 %. Based on LSU rRNA sequences, two genera, *Nosema* and *Vairimorpha*, formed a complex (Fig. 2a). From the phylogenetic tree, we can see that *Nosema* sp. HR is unique and shares the same ancestor with other species within the Nosema complex. The identities of *LSU rRNA* sequences between *Nosema* sp. HR and other species within the Nosema complex are 95–98 %. Identity between *Nosema* sp. HR and the type species of the genus, *N. spodopterae*, is 99 %. The identities between *Nosema* sp. HR and Vairimorpha species within Nosema complex are 94–96 %, but only are 79–83 % between *Nosema* sp. HR and species with the Vairimorpha complex (type species *Vairimorpha necatrix*).

Table 3 Difference of rDNA units between *Nosema* sp. HR and other microsporidian

Species	GenBank Accession No.	LSU rDNA	SSU rDNA	5S rDNA
Nosema bombycis	AY259631	17	4	4
Nosema spodopterae	AY747307	14	3	3
Nosema fumiferanae	HQ457432	25	12	3
Microsporidium 57864	U90885	573	206	24

The *SSU rRNA* of *Nosema* sp. HR consists of 1233-bp nucleotides, and the GC content is 34.06 %. The phylogenetic tree of *SSU rRNA* sequences is shown in Fig. 2b. Among the sequences in this tree, the identities of *SSU rRNA* sequences between *Nosema* sp. HR and other species within the true

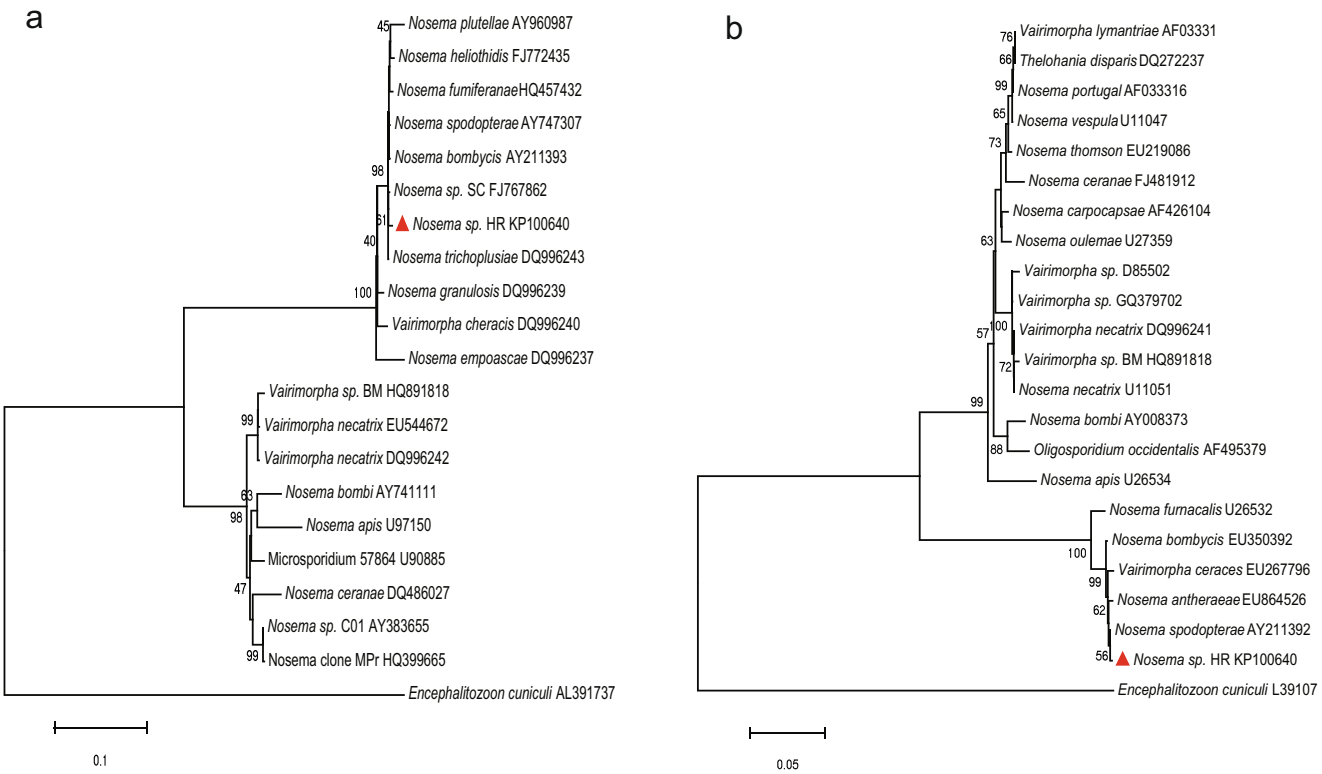

Fig. 2 Phylogenetic analysis of the *Nosema* sp. HR based on the *LSU rRNA* and *SSU rRNA* genes. **a** A phylogenetic tree based on the *LSU rRNA* sequences. **b** A phylogenetic tree based on the *SSU rRNA* sequences. Phylogenetic trees were constructed by using the neighbor-joining method. The bootstrap values are indicated at the nodes. The GenBank accession number for each sequence is given adjacent to the corresponding species name. The *Nosema* sp. HR sequence has been indicated by *red triangle* in the phylogenetic tree

Nosema complex are 98–99 %. The identities between *Nosema* sp. HR and Vairimorpha species within Nosema complex are 95–98 %, but only 84–89 % between *Nosema* sp. HR and species within the Vairimorpha complex (type species *V. necatrix*).

From phylogenetic trees of *LSU rRNA* and *SSU rRNA* sequences, they both indicate that *Nosema* sp. HR is an isolate of true Nosema. In addition, all species analyzed in these two phylogenetic trees are parasites of lepidopteran insects.

Discussion

Microsporidia infect a broad range of vertebrates and invertebrates including insects, fishes, and mammals (Weiss 2001; Casal et al. 2009; Morsy et al. 2012, 2013). And, one microsporidian can infect several host species. In this study, a new isolate, *Nosema* sp. HR, had been isolated from *H. rhodope* Cramer (Lepidoptera, Zygaenidae), which can cause serious destruction to *B. javanica* BL. in China. Usually, SSU rRNA sequence has been widely used as a molecular marker for estimating phylogenetic relationships among microsporidia; however, Canning et al. (1999) and Tsai et al. (2003) suggested that this highly conserved gene could not be used to distinguish between closely related species. So, other genetic marker was required for analyzing phylogenetic affinities. Some researchers suggested that the microsporidian *ITS rRNA* and *LSU rRNA* sequences have good potential as informative molecular markers (Tsai et al. 2005; Zhao et al. 2014). According to the results of this study, the identity of the *rRNA* gene sequences of *Nosema* sp. HR with those of the other Nosema species (such as *N. spodopterae*) is high. Phylogenetic analysis of *LSU rRNA* and *SSU rRNA* (Fig. 2a, b) revealed that this isolate is closely related to the members of the true Nosema group.

Microsporidian *Nosema* sp. HR, which isolated from the insect *H. rhodope*, not only has more similarity on sequence to *N. spodopterae* than to *V. necatrix*, but also the organization of the *rRNA* gene of *Nosema* sp. HR is 5′-LSU-ITS-SSU-IGS-5S-3′, which is same to that of *N. bombycis* (Huang et al. 2004), *N. spodopterae* (Tsai et al. 2005), *Nosema antheraeae* (Wang et al. 2006), *Nosema plutellae* (Ku et al. 2007), and uncultured Nosema (Tsai et al. 2009). However, this isolate has a few nucleotide differences to other microsporidian species (Table 3). This result further indicates that this isolate is a unique one among the Nosema complex.

According to the results of the morphological and phylogenetic characteristics of *Nosema* sp. HR, we suggest that this new microsporidian *Nosema* sp. HR belongs to the true

Nosema group. Moreover, this microsporidian can also infect *B. mori* and cause heavy loss in sericulture. So, some wild insects can carry microsporidian to infect silkworm. In sericulture, we should pay attention not only to *N. bombycis*, but also to other microsporidian (such as *Nosema* sp. HR) in the future.

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