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Identification of a microsporidian isolate from *Cnaphalocrocis Medinalis* and its pathogenicity to *Bombyx mori*

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A microsporidian, CmM2, was isolated from *Cnaphalocrocis medinalis*. The biological characters, molecular analysis and pathogenicity of CmM2 were studied. The spore of CmM2 is long oval in shape and $3.45 \pm 0.25 \times 1.68 \pm 0.18\,\mu m$ in size, the life cycle includes meronts, sporonts, sporoblasts, and spores, with typical diplokaryon in each stage, propagated in binary fission. There is positive coagulation reaction between CmM2 and the polyclonal antibody of *Nosema bombycis* (N.b.). CmM2 spores is binuclear, and has 10–12 polar filament coils. The small subunit ribosomal RNA (SSU rRNA) gene sequence of CmM2 was obtained by PCR amplification and sequencing, the phylogenetic tree based on SSU rRNA sequences had been constructed, and the similarity and genetic distance of SSU rRNA sequences were analyzed, showed that CmM2 was grouped in the *Nosema* clade. The 50% infectious concentration of CmM2 to *Bombyx mori* is 4.72×10^4 spores ml^{-1}, and the germinative infection rate is 12.33%. The results showed that CmM2 is classified into genus *Nosema*, as *Nosema* sp. CmM2, and has a heavy infectivity to *B. mori*. The result indicated as well that it is valuable taxonomic determination for microsporidian isolates based on both biological characters and molecular evidence.

Abbreviations: CmM2 – A microsporidian was isolated from *Cnaphalocrocis medinalis*; **N.b.** – *Nosema bombycis*; **SCTB** – 0.1 M sodium cacodylate trihydrate buffer; **CTAB buffer** – 100 mmol L^{-1} Tris–HCl pH 8.0, 1.4 mol L^{-1} NaCl, 20 mmol L^{-1} EDTA, 2% CTAB; **TEM** – transmission electron microscope; **PCR** – polymerase chain reaction; **SSU rRNA** – the small subunit ribosomal RNA

Keywords: Microsporidia / *Cnaphalocrocis medinalis* / Morphology / Small subunit ribosomal gene / Cross infection

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Introduction

Microsporidia, a specific group of intracellular parasites, are ubiquitously distributed throughout the animal kingdom [1], and more than 1300 species in 160 genera are currently recognized [2]. Microsporidia may be the most important group of entomopathogens of insects, and have been isolated from all insect orders, such as *Nosema*, an important genus of microsporidia among insects, infects more than 150 hosts from 12 insect orders [3]. Many microsporidia are considered to have a wide host range, the cross-infection of microsporidia often occurs between wild insects and economic insects, that has many negative effects on production capacity for the infection of microsporidia to economic insects (such as silkworm, bee, etc.) [4, 5]. Some microsporidia isolated from some wild insects are studied, such as *Laphygma exigua* [6], Fall webworm *Hyphantria cunea* [7], *Mimastra cyanura* Hope [8], *Delias pasithoe* [9], and so on, show that they all can infect silkworm, *Bombyx mori*, so they cause considerable problems in industries of sericulture. Accurate identification of these microsporidia may play an important role in prognosis and prevention assessment as well as in understanding the pathogenesis and epidemiology, which will help to control the cross-infection of microsporidia for economic insects.

Traditional taxonomic studies and species classification of the microsporidia are mainly based on biological characters such as shape and size, ultrastructure,

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life cycle, serological reaction, microsporidium–host relationships, parasitic tissue specificity as well as transmission way [10]. However, for some basic biological problems, taxonomic determinations based on these criteria alone can sometimes be problematic. For example, a microsporidian isolated from the *Cerace stipatana* (Walker) was describe as a species of *Vairimorpha* based on the ultrastructural characteristics and exhibition of octosporous sporogony in life cycle [11], but based on its SSU rRNA sequence, it is clustering in a clade with *Nosema* spp. rather than in the clade containing the *Vairimorpha* type species [12]. Nowadays, with the development of molecular biotechnology, molecular techniques have widely used in phylogenetic analysis and identification of insect pathogens, the small subunit ribosomal RNA (SSU rRNA) gene sequence of insect microsporidia is highly conserved that can be an important sequence for molecular classification of microsporidia [13]. Therefore, phylogenetic analysis of SSU rRNA gene is an important tool for the classification of microsporidian species.

Cnaphalocrocis medinalis is a migratory pest, which mainly causes damage on rice, has 9–11 generations in South China [14], and the rice field and mulberry field are cross-distribution in China, it is possible that the cross-infection of microsporidia could occur between *C. medinalis* and silkworm. So we analyzed a microsporidian which was isolated from *C. medinalis* for its taxonomic status and pathogenicity to silkworm, *B. mori*. We present a full description of the parasite morphology, life cycle, serological reaction, transmission way, parasitic tissue specificity, and peroral infection, which together with analysis of SSU rRNA sequences, have preliminarily determined the taxonomic status of a novel microsporidian isolate by taking into account both biological characters and molecular evidence, and showing the level of infection to silkworm, *B. mori*.

Materials and methods

Materials

CmM2 was originally collected from the adults of *Cnaphalocrocis medinalis* (Lepidoptera, Pyralidae) in Guangxi Zhuang Autonomous Region, China on September 3, 2012. *Nosema bombycis* (N.b.) was stored at Sericultural Research Institute of Guangxi Zhuang Autonomous Region. *B. mori* larvae (strain, 932 × Fu rong) were provided by Sericultural Research Institute of Guangxi Zhuang Autonomous Region. The rabbits (strain, New Zealand rabbits) were provided by Guangxi Medical University Laboratory Animal Centre, China.

Freund's adjuvant, proteinase K, DNA polymerase and RNaseA were purchased from Sigma–Aldrich Fine Chemicals (CMO, USA). Phenol, chloroform, isoamylol, and ethanol all were purchased from Shanghai Chemicals Factory (P. R. China). Agarose was purchased from Biowest (Spain).

Centrifugal machine (Eppendorf 5810R, Germany). Phase contrast microscope (Olympus BX51, Japan). Transmission electron microscope (TEM) (Hitachi H-600, Japan). PCR instrument (Biorad T100™ Thermal Cycler, USA). 3730xl DNA analyzer (Applied Biosystems, USA).

Purification of microsporidian spores

The spores of CmM2 and N.b. were introduced into the larvae of *C. medinalis* and *B. mori* by oral inoculation, respectively. Then, after culturing and harvesting, the spores were Percoll-purified by gradient centrifugation [15], and stored in distilled water at 4 °C.

Observation of size and shape of microsporidian spores

Fresh spores of CmM2 and N.b. were observed in phase contrast microscope at 900× magnification, then measured the long axis and short axis using digital microscope measurement and analysis system, respectively.

Serological reaction of CmM2

Preparation of 1×10^9 spores ml^{-1} concentration of N.b. in 9.0 g L^{-1} NaCl as the antigen. The male rabbits was immuned with the antigen by way of complete Freund's adjuvant immunization, and after four times inoculation, the antibody titer in serum of rabbit was up to 1:4096 detected by slide agglutination, then the antiserum of polyclonal antibody of N.b. was prepared by drew blood from the heart of rabbits. The dilution of antiserum was 1:128 for the optimal agglutination, 10 μl of spores of CmM2 was mixed with 10 μl of diluents of the antiserum of N.b. in slide with slide agglutination, laid up 10 min at 27 °C, then looked through a microscope at the condensation reaction between CmM2 and the polyclonal antibody of N.b. [16].

Observation of the life cycle of CmM2

Second instar larvae of laboratory reared silkworm were inoculated for 6 h with infective concentration of 1×10^7 spores ml^{-1} of CmM2 then smeared the posterior portion of midgut of larvae for the first time after 12 h, and smeared the posterior portion of midgut of larvae in every 12 h until new mature spores had formed. The air-dried smears were fixed in 95% methyl alcohol for 3 min

and stained for 10 min with 10% Giemsa stain buffered at pH 7.4, mounted the slides with resinene. Then looked through a microscope at the phases of development of CmM2 at 1500× magnification [17].

Observation of ultrastructure of CmM2

Preparation 1.0 ml of 3×10^8 spore ml^{-1} concentration of CmM2, centrifuged at 3600 rpm min^{-1} for 10 min. The isolated spores was pre-fixed overnight at 4 °C in 6.0% v/v glutaraldehyde in 0.1 M sodium cacodylate trihydrate buffer, pH 7.4 (SCTB), then was pre-embedded with 2.5% agar, and fixed for 12 h at 4 °C in 6.0% v/v glutaraldehyde in SCTB again, followed by washing in SCTB. The fixed sample was then post-fixed for 2 h at 4 °C in 1% aqueous OsO_4 (w/v) and washed in SCTB. Subsequently, the sample was fixed for 2 h at 4 °C in 0.5% mineral chameleon in 9 g L^{-1} NaCl solution and washed in the same solution. The sample was dehydrated in ethanol series and was finally embedded in epoxide resin, then dried, and ultrathin sections were cut with a ultramicrotome and were stained with uranyl acetate and lead citrate. Sections was examined and photographed with TEM [18].

Preparation of genomic DNA of microsporidia

Genomic DNA of CmM2 was extracted using the CTAB method [19]. Approximately 1×10^9 spores were resuspended in 0.5 ml of 2% CTAB buffer in a 1.5-ml Eppendorf tube, and incubated at 37 °C for 1 h, 1 mg of proteinase K was added and incubated overnight at 56 °C after homogenization. The sample solution was incubated at 37 °C for 0.5 h after added 0.02 mg RNaseA. Subsequently, 0.5 ml of phenol was added to the recovered aqueous phase after centrifuged at 1.2×10^4 r min^{-1} for 5 min, followed by the addition of 0.5 ml of phenol chloroform isoamyl alcohol (25:24:1 v/v) to the recovered aqueous phase after centrifuged again. 0.45 ml of chloroform extraction was performed, followed by the addition of the same volume of isopropyl alcohol, and the sample solution was stored at −20 °C for 1.0 h after homogenization, then centrifuged again, the precipitation was rinsed twice with 70% ethanol before drying in natural conditions. Then the extracted DNA was stored at −20 °C after dissolving in 30 μl of sterile water.

Multiprimer PCR and sequence of SSU rRNA gene of CmM2

Primers used in the sequencing of the small subunit ribosomal RNA (SSU rRNA) gene of CmM2 were designed based on Huang *et al.* [20] described. The primers were: 18f: 5′-CACCAGGTTGATTCTGCC-3′ and 1537r: 5′-TTATGATCCTCCTAATGGTTC-3′. The reaction system was 25 μl, and PCR was carried out as follows: after preheating of DNA at 94 °C for 8 min, followed by 28 cycles of denaturing at 94 °C for 1.0 min, annealing at 47 °C for 1 min 40 s, and extension at 72 °C for 1 min 40 s. After PCR, the aliquot from reaction was run on a 1.0% agarose gel electrophoresis with 2000 bp DNA size markers to visualize the PCR products. Gene products from three separate PCRs were pooled and sequenced directly for bidirectional sequencing, then the SSU rRNA gene sequence of CmM2 was obtained by effective montage of positive and negative sequence, and the sequence was submitted to GenBank.

Molecular phylogenetic analysis of CmM2

The SSU rRNA gene sequence of CmM2 was compared with the homologous sequences, which were obtained from GenBank through search engine (http://www.ncbi.nlm.nih.gov/BLAST). Some SSU rRNA gene sequences of *Nosema*, *Amblyospora*, *Endoreticulatus*, *Vairimorpha*, *Pleistophora*, and *Glugea* were downloaded, the phylogenetic tree based on SSU rRNA gene sequence of CmM2 and 22 sequences of other strains of microsporidia was constructed, 1000 bootstrap replications were generated to test the robustness of the trees, *Beauveria bassiana* (EU334679) was used as an out-group, as implemented by the Neighbor-Joining of MEGA5.0 software [21]. The similarity and genetic distance of SSU rRNA gene sequences of some sequences was analyzed by MegAlign of DNAStar software.

Determination of peroral infection of CmM2 to *B. mori*

B. mori larvae of laboratory reared silkworm were infected with CmM2, and N.b. was the control pathogen, to determine their pathogenicity. A series 10 times concentration of 10^3–10^7 spore ml^{-1} of CmM2 and N.b. were used as infective concentration on *B. mori* larvae that newly hatched larvae were fed with the mulberry leaf coated with 0.2 ml of each infective concentration, respectively, three replicates of 30 healthy larvae each were maintained. After consumed the leaf that coated with spores, the larvae were fed with disinfected mulberry leaf until fifth instar. Then look through a microscope at the presence of spores for each larvae, investigated the number of infected larvae. The peroral infection of CmM2 and N.b. were expressed as 50% infectious concentration through Reed–Muench method, respectively.

Determination of germinative infection of CmM2 to *B. mori*

Fourth instar larvae of laboratory reared silkworm were inoculated per os with 4.0 ml infective concentration of 1×10^5 spores ml^{-1} of CmM2 spores for 50 larvae. The

larvae were reared with mulberry leaf until mature larvae. After single moth laying and individual inspection of moth, the eggs from the heavy infected moths derived from infected larvae were selected to hatch and rear until third instar, then looked through a microscope at the presence of spores for each larvae, and investigated the rate of infected larvae.

Examination of infected larval tissues

Second instar larvae of laboratory reared silkworm were inoculated per os with infective concentration of 1×10^5 spores ml^{-1} of CmM2 spores. The larvae were fed with mulberry leaf until Day 4 fifth instar, then different tissues from infected larvae were dissected out to identify the host tissues that were infected, such as midguts, gonads, malpighian tubules, fat body, silk glands, and so on, individually washed twice in physiological saline and examined for the presence of spores using phase contrast microscopy.

Results

Shape and size of CmM2 spores

The living spores of CmM2 was long oval in shape and measured 3.45 μm ± SE 0.25 in long axis and 1.68 μm ± SE 0.18 in short axis under a phase contrast microscope (Table 1), not significantly longer and shorter than that of N.b. ($t < t_{0.05} = 2.045$; *t*-test). The refractivity of CmM2 spores was strong.

Serological reaction of CmM2 spores

The serological relationship between the spores of CmM2 and N.b. was detected by slide agglutination. A strong condensation was observed when CmM2 spores mixed with the antiserum of N.b. (Fig. 1), proved that agglutination reaction had happened between the spores of CmM2 and the polyclonal antibody of N.b., showed that the surface protein antigen of CmM2 was similar to N.b., so the genetic relationship of CmM2 and N.b. has been very close.

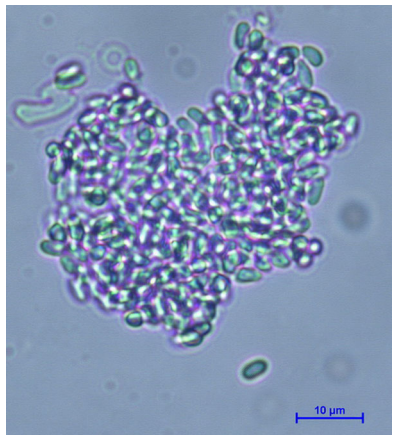

Figure 1. The coagulation reaction between CmM2 and polyclonal antibody of *Nosema bombycis* (N.b.).

Life cycle of CmM2

The developmental stages of CmM2 in the posterior portion of midgut of *B. mori* were observed (Fig. 2). The diplokaryotic meronts were observed in earliest stage (12–24 h), then the meronts developed in binary division (24–48 h), which resulted in the formation of binucleate sporonts (48–72 h). Sporoblasts were formed in the following stage (72–96 h). The short tube type spores and second infection were observed in late stage (96–120 h), and mature long tubular spores were formed in 132 h, so the life cycle of CmM2 took about 132 h, all stages were diplokaryotic. Therefore, the development type of CmM2 was accorded with the basic taxonomic features of *Nosema*.

Ultrastructure of CmM2 spores

The TEM observations revealed that the spores of CmM2 was oblong ovate and binucleate with thick wall, the wall was consisted of three layers, exospore, endospore, and cytoplasm membrane, exospore was thickened and translucent, and endospore was rugose and unlayered. The spores had 10–12 coils of the polar filament, and most spores contained 11 polar filament coils. These indicated that CmM2 had the basic taxonomic features of *Nosema* (Fig. 3).

Table 1. The shape and size[a] of CmM2 and *Nosema bombycis* (N.b.) spores.

Microsporidia	Shape	Long axis (μm)	Short axis (μm)	*t*-Value[b] of long axis between CmM2 and N.b.	*t*-Value[b] of short axis between CmM2 and N.b.
CmM2	Long oval	3.45 ± 0.25	1.68 ± 0.18	1.976	1.212
N.b.	Oval	3.01 ± 0.21	1.73 ± 0.18		

[a]The long axis value and short axis value of CmM2 and N.b. were the average values of 30 spores, respectively.
[b]The *t* values were measured by the method of *t*-test.

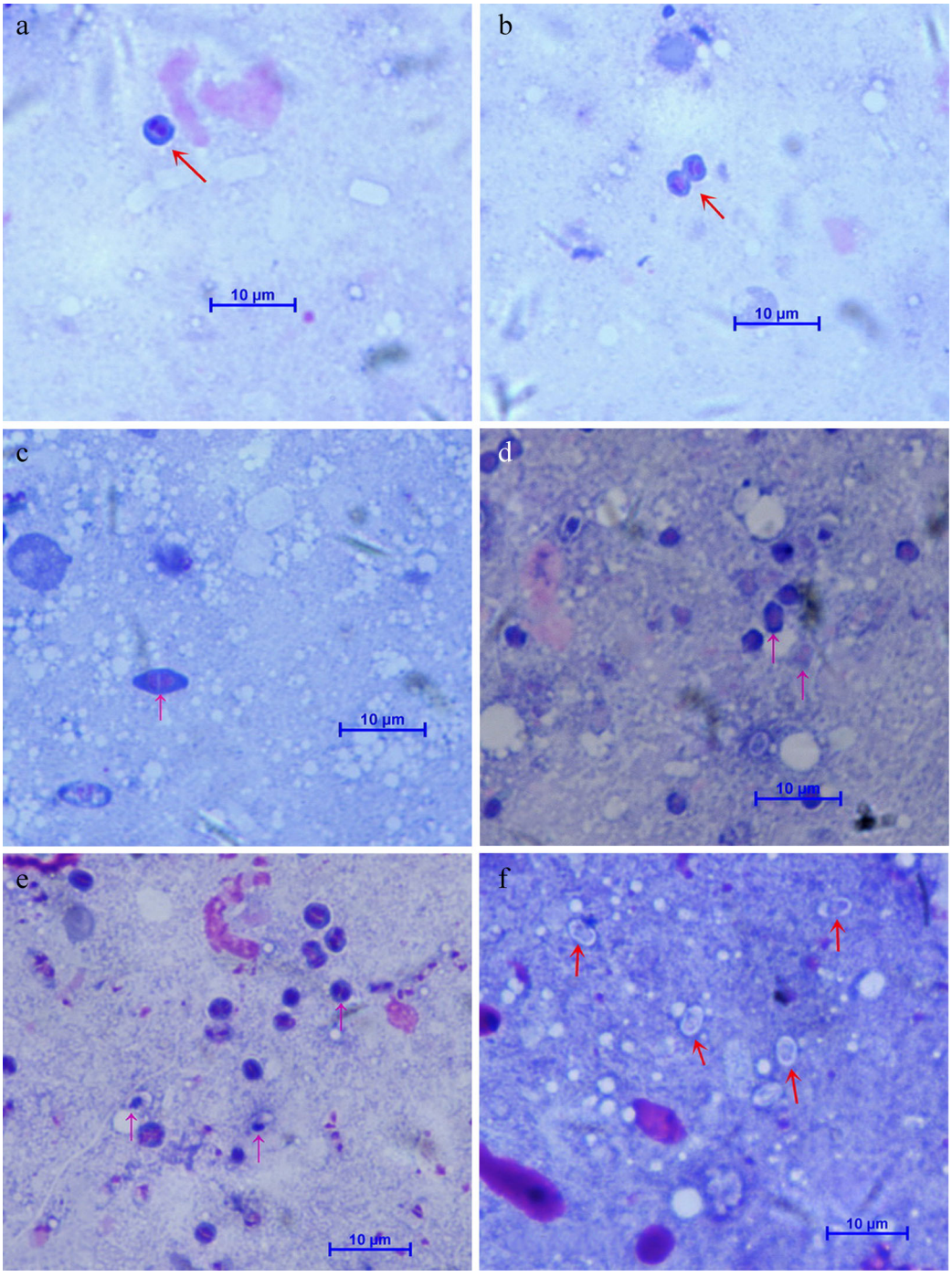

Figure 2. Developmental stages of CmM2 in midgut tissue of *Bombyx mori*. Giemsa stained. (a) Binuclear meronts (12–24 h); (b) division of meronts (24–48 h); (c) formation of binucleate sporonts (48–72 h); (d) sporoblasts (72–96 h); (e) short tube type spore and second infection form (96–120 h); (f) mature long tubular spores (132 h).

Phylogenetic analysis of CmM2

The SSU rRNA gene sequence of CmM2 was deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) under the Accession Number KC836092, the SSU rRNA gene sequence was 1150 bases long, the base composition of this molecule was 33.13% GC. We compared the SSU rRNA gene sequence of CmM2 with the corresponding rRNA gene regions in other 22 species

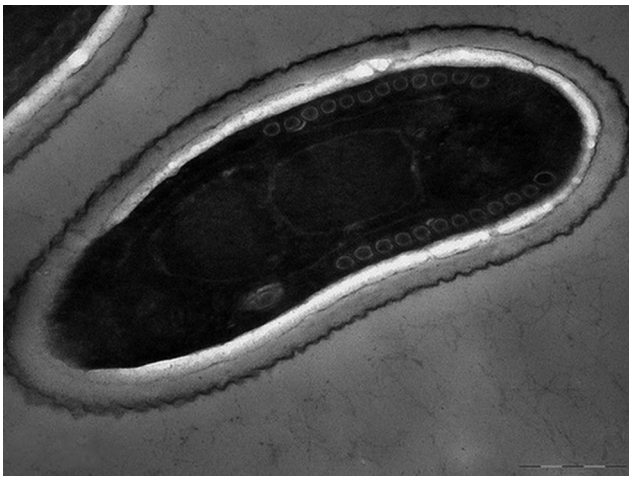

Figure 3. Ultrastructure of spores of CmM2.

of seven microsporidian genera, phylogenetic analysis of the aligned sequences was carried out by using MEGA5.0 program and the phylogenetic tree constructed by Neighbor-Joining was shown in Fig. 4. The phylogenetic tree revealed that the microsporidia of each genera was clustered in each clades with more than 98% bootstrap support, so the phylogenetic tree that we constructed was valuable. CmM2 was grouped in the *Nosema* clade, indicated that CmM2 was belong to the genus *Nosema*.

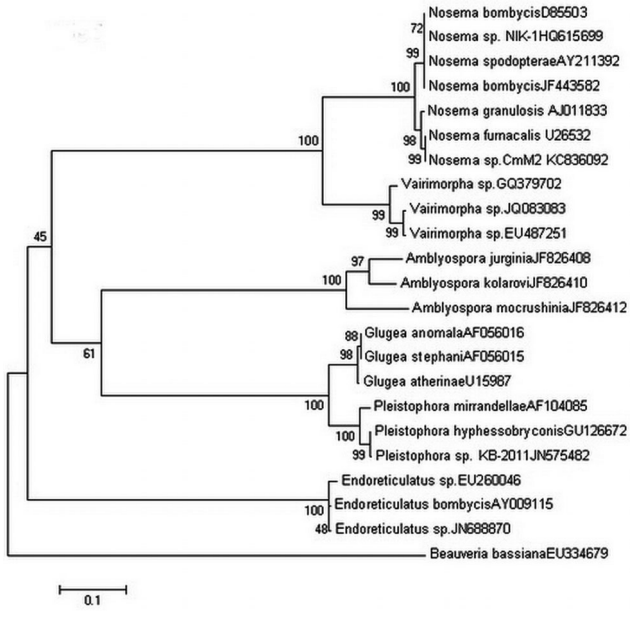

Figure 4. Phylogenetic tree construct by the Neighbor-Joining method base on the SSU rRNA gene sequences of 23 species of microsporidia. *Beauveria bassiana* (EU334679) is used as the outgroup. The tree used 1000 bootstrap replicates. The numbers on the tree represents the percentage of bootstrap values.

In order to further examined the relationships among CmM2 and other microsporidian species, the similarity and genetic distance of SSU rRNA gene sequences of CmM2 and other 14 microsporidian species were compared (Table 2), indicated that the SSU rRNA genes of microsporidia had high identities and less divergence among CmM2 and *Nosema* species, the similarity of CmM2 and *Nosema furnacalis* (U26532) was 99.9%, and their genetic distance was only 0.1, and had lower identities and higher divergence between CmM2 and some microsporidian species in other genera, such as *Endoreticulatus*, *Amblyospora jurginia*, and *B. bassiana*.

Therefore, molecular analysis indicated that CmM2 was belong to the genus *Nosema*, we temporarily named it as *Nosema* sp. CmM2, and *Nosema* sp. CmM2 was closely related to *Nosema furnacalis* (U26532).

Although the SSU rRNA gene sequence for *Nosema* sp. CmM2 has already been sequenced for part (GenBank Accession No. KC836092), here we sequenced the SSU rRNA gene of a strain of *Nosema medinalis* for the first time.

Peroral infection of *Nosema* sp. CmM2 to *B. mori*

The pathogenicity of microsporidia was determined with 50% infectious concentration (IC_{50}). The IC_{50} values of *Nosema* sp. CmM2 and N.b. were 4.72×10^4 and 2.49×10^4 spores ml^{-1}, respectively, indicated that *Nosema* sp. CmM2 was found to be the minor virulent as compared to N.b., but *Nosema* sp. CmM2 still had heavy peroral infection to *B. mori*.

Transovarial transmission of *Nosema* sp. CmM2

To check for transovarial transmission of *Nosema* sp. CmM2 to *B. mori*, the third instar of second generation of the infected mother moths were examined. The result showed that the incidence of transovarial transmission of *Nosema* sp. CmM2 was 12.33%, so *Nosema* sp. CmM2 had a transovarial transmission to *B. mori*.

Tissues infected

In fifth instar larvae of *B. mori*, the tissue specificity of infection was examined for the spores of *Nosema* sp. CmM2. The tissues of midguts, gonads, malpighian tubules, fat body, and silk glands all were heavily infected by spores of *Nosema* sp. CmM2, showed that *Nosema* sp. CmM2 invaded all the tissues examined. Therefore, *Nosema* sp. CmM2 had a systemic infection to silkworm, *B. mori*.

Table 2. The similarity and genetic distance[d] of SSU rDNA gene sequence between 15 microsporidian strains[c].

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		99.9	98.7	96.3	96.7	96.7	96.6	96.6	96.1	96.6	95.1	95.9	47.0	43.0	37.7
2	0.1		98.8	96.4	96.9	96.9	96.8	96.8	95.3	96.8	94.6	96.2	44.7	42.9	36.4
3	0.8	1.0		96.6	97.0	97.0	97.1	97.1	96.6	97.1	95.2	96.4	46.3	45.0	37.3
4	1.2	1.3	1.4		97.7	97.4	97.7	97.8	97.2	97.5	95.1	97.3	48.3	44.0	36.1
5	1.9	1.7	2.0	1.1		100.0	99.8	99.9	98.5	99.9	97.7	99.4	47.1	42.3	36.3
6	1.9	1.7	2.0	1.1	0.0		99.8	99.9	98.4	96.8	64.0	99.4	46.3	39.9	18.5
7	1.9	1.9	1.9	1.1	0.2	0.2		99.9	98.5	99.9	97.6	99.4	47.2	42.3	36.3
8	1.9	1.8	1.9	1.1	0.1	0.1	0.1		98.6	100.0	97.6	99.5	47.1	42.3	36.3
9	2.0	2.3	2.0	1.5	0.5	0.5	0.4	0.4		98.5	96.4	98.0	47.6	44.0	36.8
10	1.9	1.8	1.9	1.1	0.1	2.2	0.1	0.0	0.4		94.3	99.5	46.3	39.8	18.0
11	2.1	2.0	2.2	1.4	0.3	3.3	0.5	0.4	0.8	3.4		97.2	47.7	39.3	16.8
12	2.5	2.3	2.4	1.6	0.6	0.6	0.6	0.5	0.9	0.5	0.7		46.7	42.1	36.0
13	57.8	57.3	58.3	57.7	57.4	52.1	57.4	57.4	58.1	51.2	51.6	57.4		50.1	33.1
14	51.6	50.9	50.9	50.8	49.4	50.2	49.4	49.4	49.9	50.3	49.0	49.4	43.6		34.5
15	88.9	87.8	88.5	88.7	89.0	91.1	88.5	88.6	90.6	90.3	92.4	89.9	114.9	115.1	

[c]1, *Nosema* sp. CmM2 (KC836092); 2, *Nosema furnacalis* (U26532); 3, *Nosema granulosis* (AJ011833); 4, *Nosema* sp. GKK-2009 (GQ337009); 5, *Nosema bombycis* strain GD 1 (JF443582); 6, *Nosema bombycis* (AY259631); 7, *Nosema bombycis* (D85503); 8, *Nosema spodopterae* (AY211392); 9, *Nosema pyrausta* (AY958071); 10, *Nosema heliothidis* (FJ772435); 11, *Nosema fumiferanae* (HQ457432); 12, *Nosema antheraeae* (EU864526); 13, *Endoreticulatus* sp. (AY502945); 14, *Amblyospora jurginia* (JF826408); 15, *Beauveria bassiana* (EU334679).
[d]Data in upper right half of the table are of sequence similarity (%), and the bottom left half are of genetic distance.

Discussion

The traditional taxonomy of microsporidian has long been established on the basis of spore morphology, including ultrastructural and functional characteristics, as well as on host range and developmental life cycle in a particular host [22, 23]. The diplokaryotic nucleus, the disporoblastic sporogony, mainly infected in the host cell cytoplasm, oval in shape for matured spores, two nuclei in all phases of development of life cycle and transovarial transmission all are characteristic of the genus *Nosema* [24, 25]. *Nosema* species can infect a host by ingestion, by vertical transmission, and possibly by insertion into the hemolymph by a vector [26].

In this study, the life cycle, morphology, serological relationships, and pathogenicity of *Nosema* sp. CmM2 were observed. The shape of meronts, sporons, sporoblasts, and spores all were deserved in development of life cycle of *Nosema* sp. CmM2, two nuclei in each stages, and propagated in binary fission. The spores were oval in shape, and was binucleate, which was consistent with the diplokaryon in life cycle, the number of polar filament coils was between 10 and 12. There was positive condensation reaction between the spores of *Nosema* sp. CmM2 and the polyclonal antibody of N.b. *Nosema* sp. CmM2 had transovarial transmission and systemic infection to type species of lepidopteran hosts, *B. mori*. Therefore, *Nosema* sp. CmM2 had the biological characters substantially similar to the type species, N.b. [22], it belong to genus *Nosema*.

Further studies on molecular phylogenetic analysis of *Nosema* sp. CmM2 will strengthen the taxonomic status, which we have based on biological characters. Comparative studies of SSU rRNA gene have been shown to be very useful in microsporidian taxonomy and phylogeny [27], and SSU rRNA gene also can be used as a molecular marker for estimating relationships among microsporidia [28]. The phylogenetic tree, similarity and genetic distance of SSU rRNA gene sequences of *Nosema* sp. CmM2 and other microsporidia showed that *Nosema* sp. CmM2 was grouped in the *Nosema* clade; the GC content of SSU rRNA gene sequences of *Nosema* sp. CmM2 was 33.13%, it was similar to that of *Nosema* species of the "true-*Nosema*" group (32.88–33.56%) [29]. So *Nosema* sp. CmM2 was a *Nosema* species, and had closely related to *N. furnacalis* (U26532).

Deeper insights into the biology and evolutionary relationships of the microsporidia will lead to a formal description of each microsporidian species, and molecular data alone cannot replace detailed cytological observations and associated conclusions, if evaluated correctly, will lead to similar phylogenetic conclusions [30]. In this paper, we had the similar determination for the taxonomic status of *Nosema* sp. CmM2 that it was belonging to the genus *Nosema* based on the biological characters and molecular data. We proposed that the parasite described here represents a novel species, *N. medinalis*. The results indicated as well that it is valuable to elucidate phylogenetic relationships and taxonomic status of microsporidian species by taking

into account both biological characters and gene sequence data.

N.b. is the typical pathogen of microsporidiosis for silkworm, *B. mori* which has caused heavy losses in sericulture in Europe in the middle of 19th century. The pathogenicity of *Nosema* sp. CmM2 to silkworm, *B. mori* was studied, showed that the 50% infectious concentration of *Nosema* sp. CmM2 and N.b. were in the same grade, all were 10^4 spores ml^{-1}, and *Nosema* sp. CmM2 had a systemic infection to *B. mori*, which similar to N.b., invaded all the tissues examined, especially the gonadal infection has an important bearing on the vertical transmission [4], the transovarial transmission rate of *Nosema* sp. CmM2 to silkworm was 12.33%, which has a great influence on the next generation of infected mother moths. So the infection and transovarial transmission of *Nosema* sp. CmM2 in the silkworm were evident. The scales from lepidopteran insects infected with microsporidia are often contaminated with mature spores, and the feces and scales of insects containing parasite spores might stick to mulberry leaves and transmit microsporidia infections to silkworm larvae [31]. *C. medinalis* is a migratory pest [14], and the rice field is near the mulberry field and silkworm farm, it is possible that *N. medinalis* can be transmitted to infect silkworm larvae when the feces, scales, and dead bodies of hosts stick to mulberry leaves. Therefore, the microsporidian isolated from *C. medinalis* had a cross infection to *B. mori*.

The present study revealed that under normal sericultural practices, the microsporidiosis of silkworm might not be exclusively caused by N.b., it is possible that the pathogens were transmitted by wild insects, such as *C. medinalis*, and that species certification of the pathogen for occur cross-infection to silkworm research is important. Therefore, the research of phylogenetic relationship and pathogenicity of microsporidian isolated from *C. medinalis* have practical value on the prevention of microsporidiosis for silkworm, *B. mori*.

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Conflict of Interest Statement

We declared that there are not any financial conflicts of interest for this article, and the original authors and source are credited.

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