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SHORT COMMUNICATION

Identification of a microspordium isolated from *Megacopta cribraria* (Hemiptera: Plataspidae) and characterization of its pathogenicity in silkworms

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Abstract A new microsporidium isolated from *Megacopta cribraria* was characterized by both biological characteristics and phylogenetic analysis. Moreover, its pathogenicity to silkworms was also studied. The spores are oval in shape and measured 3.64 ± 0.2 × 2.20 ± 0.2 μm in size. Its ultrastructure is characteristic of the genus *Nosema*: a diplokaryon, 13–14 polar filament coils and posterior vacuole. Its life cycle includes meronts, sporonts, sporoblasts and mature spores, with a typical diplokaryon in each stage and propagation in a binary fission. A phylogenetic tree based on SSU rRNA and rRNA ITS gene sequence analysis further indicated that the parasite is closely related to *Nosema bombycis* and should be placed in the genus *Nosema* and subgroup 'true' *Nosema*. Furthermore, the microsporidium heavily infects lepidopteran silkworm insect and can be transmitted per os (horizontally) and transovarially (vertically). Our findings showed that the microsporidium belongs to the 'true' *Nosema* group within the genus *Nosema* and heavily infects silkworms. Based on the information obtained during this study, we named this new microsporidium isolated from *M. cribraria* as *Nosema* sp. MC.

Keywords Microsporidium · *Megacopta cribraria* · Morphology · Phylogenetic analysis · Pathogenicity

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Introduction

Microsporidia are a large group of obligate intracellular parasitic fungi that can infect both vertebrates and invertebrates (particularly insects) (Keeling 2009). To date, more than 1,300 species that belong to 160 genera have been formally described (Wittner and Weiss 1999). Among them, the genus *Nosema* is the most diverse, containing approximately 200 recognized species. Apart from lepidopteran insects (Tsai et al. 2003), microsporidia can also infect Hymenoptera (Fries et al. 1996), Amphipoda (Terry et al. 1999), Neuroptera (Bjornson et al. 2013) and Coleoptera (Zhu et al. 2011), which is indicative of their broad hosts range. *Nosema bombycis*, which causes pébrine disease in sericulture, is the type species of the genus *Nosema*.

Sericulture is a principal source of income for farmers in many developing countries, such as China, India, Brazil and Thailand. Cocoon production in China accounts for approximately 80 % of worldwide

production (Jiang and Xia 2014). However, sericulture has faced challenges from silkworm disease. Recently, pébrine disease has been spreading in China and has brought about devastating economic losses (Pan et al. 2013). Some microsporidia from other wild insects (especially Lepidoptera) were considered to be the causative organisms (Yang et al. 2007; Huang et al. 2011). Nevertheless, not all microsporidia from wild insects infect silkworms and their pathogenicity can differ substantially (Zheng et al. 2003). Thus, the identification of additional microsporidia species may help in prognosis and prevention assessments, and in understanding microsporidia-related pathogenesis and epidemiology, all of which will help control the cross-infection of economically important insects by microsporidia (Huang et al. 2013).

The traditional taxonomy of microsporidia is based on their ultrastructural features, including size and spore morphology, the number of coils of the polar tube, the developmental life cycle and the host–parasite relationship (Sprague et al. 1992). However, these criteria alone may be inadequate in some cases, especially for species determination of microsporidia with quite similar biological characteristics. Recently, SSU rRNA has been widely used as a molecular marker for this purpose and has shown marked superiority (Ku et al. 2007; Ma et al. 2008). For example, *Vairimorpha imperfecta* and the German isolate of *Vairimorpha* from the host *Plutella xylostella* were described as *Vairimorpha* based on morphological characteristics, but phylogenetic analyses of SSU rRNA sequences revealed that they are more closely related to the *Nosema* species from lepidoptera than the *Vairimorpha* species (Canning et al. 1999).

Megacopta cribraria, which mainly causes damage to lablab beans, soybeans and other legumes, is a polyphagous agricultural pest (Jenkins et al. 2010). We isolated a novel microsporidium from *M. cribraria* that was collected around a mulberry field. In this study, we determined its taxonomic status based on classical parasite morphology, life cycle, ultrastructural features and host–parasite relationship parameters, and also on our analysis of SSU rRNA and rRNA ITS gene sequences. Additionally, the pathogenicity of the isolate to silkworms was studied. This is the first formal description of a microsporidium from *M. cribraria*.

Materials and methods

Materials

Megacopta cribraria was collected in a string bean field around a mulberry field in Guangzhou, China. *Nosema bombycis* was preserved at the Sericulture and Agri-Food Research Institute, Guangdong Academy of Agricultural Sciences. The silkworm larvae (strain, Yan 7) was provided by the Sericulture and Agri-Food Research Institute, Guangdong Academy of Agricultural Sciences.

Purification of microsporidia spores

Infected *M. cribraria* were homogenized, filtered through three layers of cheesecloth and centrifuged at $3{,}000\times g$ for 15 min. Then, the pellets were resuspended in sterile water and further purification was carried out as described previously (Tsai et al. 2009).

Light and electron microscopy

The purified spores ($n = 30$) were measured under a light microscope (Olympus CX41) with an ocular micrometer and photographed with a Microscope USB Camera.

Additionally, spore samples were prepared for transmission electron microscopy (TEM). Electron microscopy was performed as previously described (Xing et al. 2011). Samples were fixed in 6 % (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C overnight and encapsulated with 1.5 % agarose solution. Then, the embedded samples were rinsed in cacodylate buffer, fixed in 1 % aqueous osmium tetroxide (w/v) for 2 h, followed by postfixation with 0.5 % potassium permanganate (w/v) for 1 h and dehydration through an ascending ethanol series. Finally, samples were embedded in Epon 812-Araldite (Fluka, Switzerland). Ultrathin Sections (60–100 nm) were cut and stained with 2 % (w/v) uranyl acetate in 50 % ethanol followed by Reynold's lead citrate. The stained grids were examined and photographed using a TECNA I G^2 12 electron microscope with an accelerating voltage of 80 kV.

Observation of the life cycle

The 2nd instar silkworm larvae were inoculated with mulberry leaves coated with a dose of 10^7 spores ml^{-1}. The posteriors of the midgut of the silkworm larvae were collected and smeared every 12 h until new spores developed. The smears were air-dried, fixed in methanol for 5 min and then stained with a 10 % (w/v) Giemsa-stain solution pH 7.2 for 20 min. The different developmental stages of the spores were observed using an oil immersion lens under an Olympus CX41 microscope.

DNA extraction and rRNA gene sequencing

DNA extraction from mature spores was carried out using the following procedure modified from Liu et al. (2004). Purified spores (10^8–10^9 spores ml^{-1}) were centrifuged at 3,000×*g* for 5 min followed by incubation with CTAB extraction buffer at 65 °C for 50 min and protease K at 56 °C overnight. Then, nucleic acids were extracted with an equal volume of chloroform and precipitated with isopropanol at −20 °C for 60 min. The DNA pellet was air dried and then dissolved in water.

The primer sets used for rRNA gene amplification were described by Huang et al. (2004). We chose 18f (5′-CACCAGGTTGATTCTGCC-3′) and 1537r (5′-TAATGATCCTCCTAATGGTTC-3′) to amplify SSU rRNA gene sequences and ILSUF (5′-TGGG TTTAGACCGTCGTGAG-3′) and S33R (5′-TAATG ATCCTCCTAATGGTTC-3′) to amplify rRNA ITS gene sequences. The PCR reactions were performed using a 2720 Thermal Cycler PCR (Applied Biosystems) in a 25 μL PCR reaction system. The PCR program: 94 °C for 5 min followed by 94 °C for 30 s, annealing temperature (47 and 56 °C, respectively) for 30 s and 72 °C for 60 s in each cycle, for a total of 30 cycles. Amplified products were detected on a 1.5 % agarose gel with Goldview staining (0.005 %, v/v). PCR products of the expected size, ∼1200 and ∼500 bp in length, were purified using a Cycle-Pure Kit (Omega Bio-Tek) and were subsequently cloned into a pMD18-T vector using the pMD^{TM}18-T Vector Cloning Kit (Takara Corp.). Three cloned DNA inserts were sent to Beijing Liuhe Huada Genomics Technology Co., LTD. for sequencing.

Molecular phylogenetic analysis

SSU rRNA gene sequences used for phylogenetic analysis were obtained from the NCBI GeneBank database. The sequences were aligned using the CLUSTAL X 1.83 program (Thompson et al. 1997). The SSU rRNA gene sequence of *Encephalitozoon cuniculi* was used as an out-group. A phylogenetic tree based on SSU rRNA gene sequences was constructed using the maximum likelihood (ML) method with the MEGA 4 program (Tamura et al. 2007). 1,000 bootstrap replicates were used to test the robustness of the trees.

Determination of peroral infection of the pathogen to the silkworms

Newly molted second instar larvae were randomly selected and placed in a 28-well plastic box, one larva per well. Fresh mulberry leaves were cut into 1 cm diameter round pieces and spore suspensions at concentrations of 1×10^4, 1×10^5, 1×10^6 and 1×10^7 spores ml^{-1} were spread evenly on the surface of leaf discs using the bulb end of a Pasteur pipette, with 5 μl per disc. The larvae were fed leaves and only those larvae that consumed all of the leaf pieces were retained. Each group was replicated three times and each repeat consisted of 30 larvae. Infectivity was determined by microscopic examination at 12 dpi and the 50 % infectious concentration (IC_{50}) was calculated using the Reed–Muench method (Reed and Muench 1938).

Tests for transovarial transmission

Oral infection of the silkworms was carried out at the fourth instar stage. Female moths obtained from exposed larvae were paired with infected males. After ovipositing, moths were inspected individually and eggs from heavily infected moths were selected for hatching. Newly hatched larvae originating from the same infected female were homogenized together, smeared on slides and observed microscopically.

Observation of parasitized tissues

Newly molted larvae were fed mulberry leaves containing 1×10^7 spores ml^{-1} of the microsporidia. Ten days later, larvae were dissected and the host

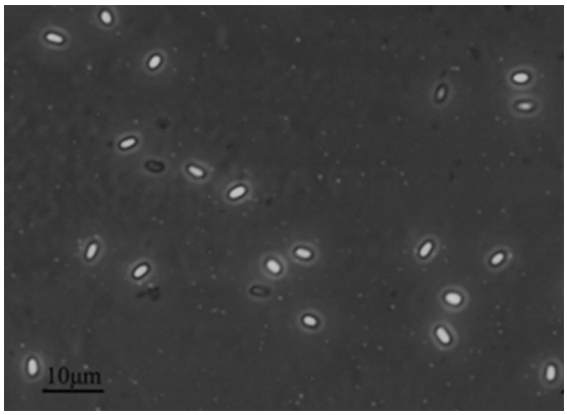

Fig. 1 Mature spores under a light microscope. Scale bar = 10 μm

tissues, such as midguts, silkglands, gonads, malpighian tubules, muscle and fat bodies, were identified individually. Each sample was washed twice in physiological saline and observed under a light microscopy.

Results

Light and electron microscopy

The spores were oval and measured 3.64 ± 0.2 × 2.20 ± 0.2 μm in size (mean ± standard error of the mean; n = 30) (Fig. 1). Longitudinal sections of the spore under a transmission electron microscope showed that the spore wall was approximately 130 nm and consisted of an electron-dense exospore with a wavy outline, an electron-lucent endospore composed of chitin and protein, and an inner cytoplasm membrane. The anchoring disk lay in the anterior extremity of the spore and connected with the straight anterior region of the polar filament. The polar filament extended laterally over the compact membranes of the polaroplast and gradually formed coils. The filament coils were comprised 13–14 isofilar turns, and always occurred in a single rank close to the spore wall. The dikaryotic nuclei were slightly separated from each other and showed a longitudinal arrangement. A membrane-bound vacuole with amorphous content was located at the posterior end of the spore (Fig. 2). These features are most closely aligned with the genus *Nosema* (Larsson 1988).

The life-cycle of a microsporidium

The developmental stages of the microsporidium in the midgut of silkworms were observed from day 0 to 6. An even number nuclei could be seen in all developmental stages (Fig. 3). A binucleate sporoplasm first appeared at 6 h after inoculation (Fig. 3a). Merogonial stages mainly occurred at 24–72 h. Spherical or ovoid binucleate meronts appeared in the host cell cytoplasm at 24 h (Fig. 3b) and the meronts increased in number by binary fission (Fig. 3c). Then, the meronts were transformed directly into binucleate sporonts (Fig. 3d). Compared to meronts, the sporogonial stages had less intensely stained cytoplasms. Spindle or pear-shaped sporonts (Fig. 3e), pear-shaped short polar tube type spores (Fig. 3g), secondary infection forms (Fig. 3h) and empty spores (Fig. 3i) were observed in this period. Sporonts (Fig. 3j) could be divided into two binucleate sporoblasts in a binary fission manner (Fig. 3k). Finally, one sporoblast formed one mature spore (Fig. 3l).

Phylogenetic analysis

The SSU rRNA gene sequence of the microsporidium was deposited at the GeneBank database (Accession number: KJ494249). The amplified SSU rRNA gene sequence was 1232 bp long, with ~34.17 % G+C content. The similarity and genetic distance of the SSU rRNA gene sequence of the pathogen and another 14 microsporidian species are shown in Table 1. The microsporidium exhibited high identities with 'true' *Nosema* species (98.5–99.9 %) and it may be most closely related to *N. bombycis* Chongqing isolate (CQ-4, Accession Number: EU350392) and *N. bombycis* Guangdong isolate (GD-5, Accession Number: JF443586). Besides, there were ~99.8, ~99.6 and ~99.5 % sequence identities with *N. bombycis* GD-1 (Accession Number: JF443582), *N. bombycis* GX-1 (Accession Number: JF443577) and *N. bombycis* (Accession Number: EU964525), respectively. The microsporidium differed from *N. Bombycis* CQ-4 by one transversion mutation at position 824 A/T and *N. Bombycis* GD-5 by three diagnostic substitutions at positions 408 C/A, 824 A/T and 1198T/G.

To better clarify the relationship among the *M. cribraria* isolate and other microsporidian species, a ML analysis based on SSU rRNA sequences was performed (Fig. 4). In the phylogenetic tree, the *M.*

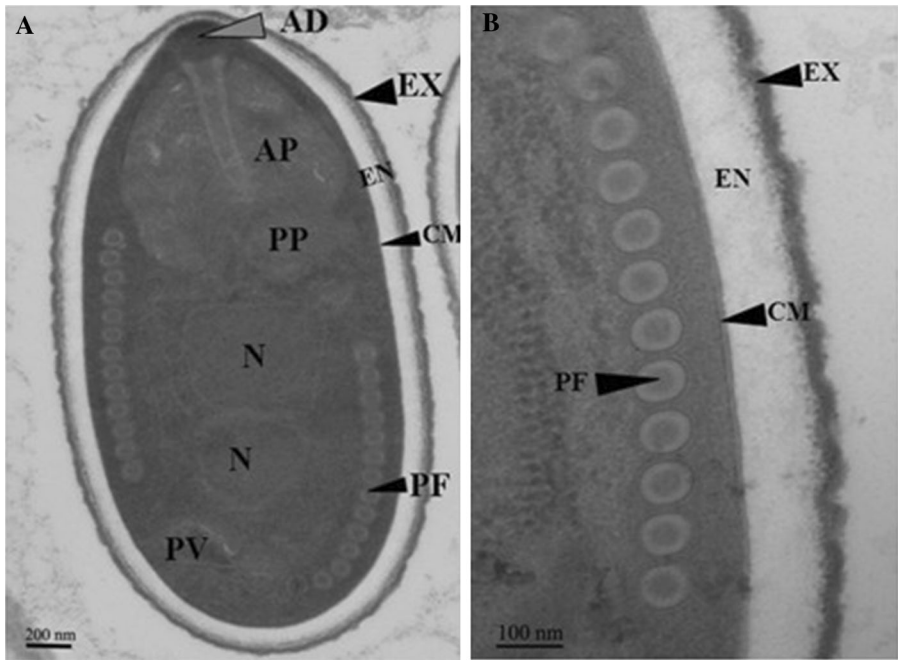

Fig. 2 An electron micrograph of a longitudinal section of a *Nosema* sp. MC spore. **a** The anchoring disk (AD), anterior polaroplast (AP), posterior polaroplast (PP), exospore (EX), endospore (EN), cytoplasmic membrane (CM), nucleus (N), polar filament (PF) and posterior vacuole (PV) are visible. Scale bar = 200 nm; **b** An electron-dense exospore with a wavy outline, an electron-lucent endospore and an inner cytoplasm membrane. The filament coils were arranged in a single layer. Scale bar = 100 nm

cribraria isolate was clustered into the *N. Bombycis* group and was found to be most closely related to *N. Bombycis* CQ-4 and *N. Bombycis* GD-5.

The rRNA ITS fragment was also obtained by PCR amplification. The rRNA ITS sequences of the *M. cribraria* isolate spanned a 181-bp length with ~17.13 % G+C content (Accession number: KJ494250). A BLAST search revealed that the isolate was most closely related to *N. bombycis* GX-18 (Accession number: JF443617) and exhibits ~97.2 % sequence identity. Notably, there was low sequence similarity (91.1 and 92.5 %) to *N. bombycis* CQ-4 and *N. bombycis* GD-5.

On the basis of both analyses, the *M. cribraria* isolate was identified as a new species of the genus *Nosema*; we named it *Nosema* sp. MC.

Peroral infection of *Nosema* sp. MC

At 12 dpi, each silkworm was homogenized and examined by microscopy. Then, the infection rates for different spore concentrations were calculated. According to the Reed–Muench method, the IC_{50} value of the silkworms against *Nosema* sp. MC was determined to be 1.24×10^5 spores ml^{-1}, while that of *N. bombycis* was 0.85×10^5 spores ml^{-1}. These results indicated that *Nosema* sp. MC had robust infection ability.

Transovarial transmission

To verify the transovarial transmission of *Nosema* sp. MC to silkworms, the newly-hatched larvae of the second generation of the infected females were examined. The results showed that the rate of transovarial transmission of *Nosema* sp. MC was nearly 60 % but the infection intensity appeared to be lower than *N. bombycis*.

Parasitized tissues

Tissue tropism for *Nosema* sp. MC was determined. Preliminary observation showed that the microsporidium is a systemic pathogen; tissues including

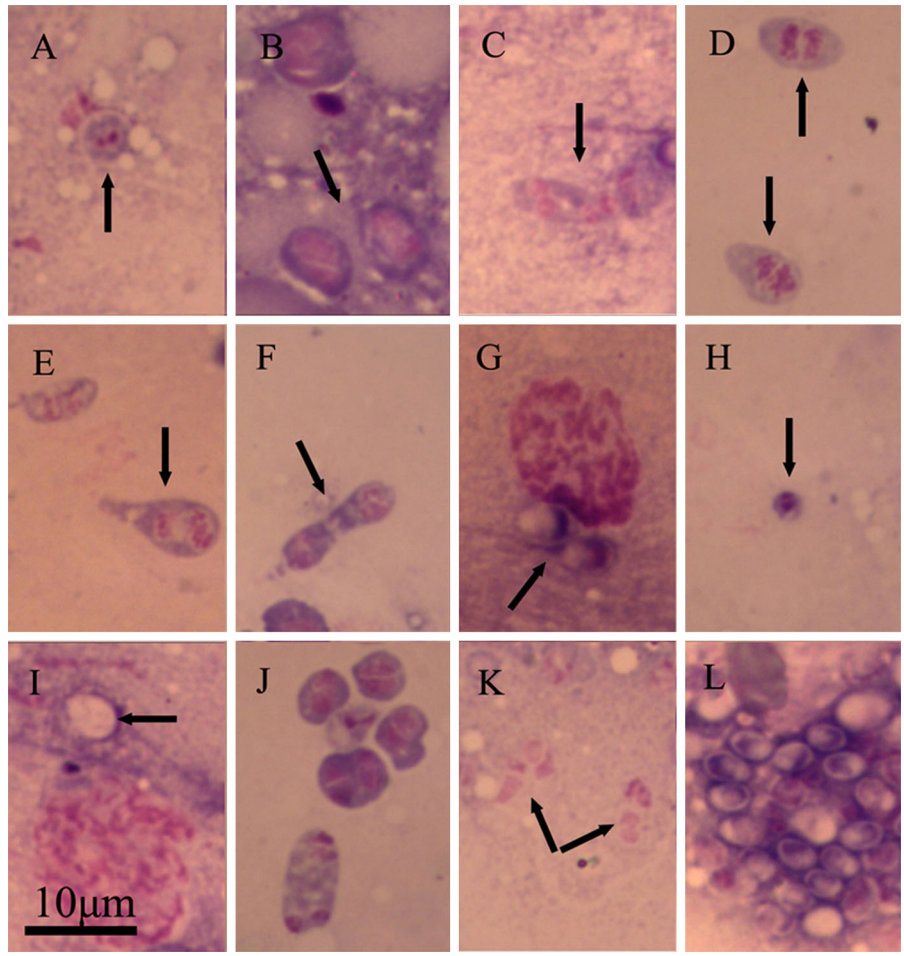

Fig. 3 The life-cycle of *Nosema* sp. MC from Giemsa-stained smears of silkworm midguts. **a** binucleate sporoplasm; **b** binucleate meronts; **c** meront binary fission; **d** two spindle sporonts; **e** spindle and pear-shaped sporonts; **f** sporont binary fission; **g** Pear-shaped short polar tube type spore; **h** second infection form; **i** empty spore; **j** bi- and tetranucleate meronts and tetranucleate sporont; **k** sporoblast and **l** mature spore

midguts, silkglands, gonads, muscle, fat bodies and Malpighian tubules were infected.

Discussion

The traditional taxonomy of microsporidia usually follows the system established by Sprague (1977). Morphological characteristics, life-cycle stages and parasite–host relationships are the major criteria used to identify microsporidia. In our study, the spores were oval in shape and measured $3.64 \pm 0.2 \times 2.20 \pm 0.2$ μm. The ultrastructure of *Nosema* sp. MC indicated that the spore nucleus is dikaryotic and has about 13 polar filament coils of the same type. Additionally, the life-cycle of *Nosema* sp. MC in *Bombyx mori* was also characterized. Although *B. mori* was used as a laboratory host and there could be some minor differences in morphology and other biological characteristics of the microsporidium, it would be helpful to determine the genus of *Nosema* sp. MC. We found that the meronts, sporons, sporoblasts, short polar tube type spores and mature spores could be observed in the developmental stages, with a typical diplokaryon in each stage and binary fission-mediated propagation. Additionally, *Nosema* sp. MC was transovarially transmitted and systemically infected *B. mori*. Therefore, the biological characteristics of *Nosema* sp. MC corresponded to the genus *Nosema* (Xing et al. 2011).

Table 1 The similarity and genetic distance of SSU rDNA gene sequences between 15 microsporidian strains

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	–	99.9	99.8	99.7	99.7	99.7	99.6	99.5	99.4	99.2	98.5	85.1	85.1	84.4	66.6
2	0.1	–	99.8	99.8	99.8	99.8	99.7	99.6	99.4	99.3	98.5	85.1	85.1	84.4	66.4
3	0.2	0.2	–	99.6	99.6	99.6	99.6	99.4	99.3	99.1	98.4	84.9	84.9	84.3	66.3
4	0.3	0.2	0.4	–	100.0	100.0	99.9	99.8	99.5	99.5	98.5	85.3	85.1	84.3	66.3
5	0.3	0.2	0.4	0.0	–	100.0	99.9	99.8	99.5	99.5	98.5	85.3	85.1	84.3	66.3
6	0.3	0.2	0.4	0.0	0.0	–	99.9	99.8	99.5	99.5	98.5	85.3	85.1	84.3	66.3
7	0.4	0.3	0.4	0.1	0.1	0.1	–	99.8	99.4	99.4	98.5	85.2	85.1	84.3	66.4
8	0.5	0.4	0.6	0.2	0.2	0.2	0.2	–	99.5	99.7	98.5	85.1	85.1	84.4	66.3
9	0.7	0.6	0.7	0.5	0.5	0.5	0.6	0.5	–	99.2	98.5	84.7	85.1	84.6	66.3
10	0.9	0.8	1.0	0.6	0.6	0.6	0.7	0.4	0.9	–	98.3	84.5	85.1	83.9	66.6
11	1.6	1.5	1.6	1.5	1.5	1.5	1.6	1.5	1.5	1.9	–	84.0	84.3	84.1	65.9
12	17.5	17.6	17.8	17.6	17.6	17.6	17.7	17.8	17.8	18.2	18.8	–	98.4	94.4	66.8
13	17.0	17.1	17.3	17.0	17.0	17.0	17.1	17.1	17.1	17.5	18.1	1.5	–	95.0	67.9
14	18.0	18.0	18.2	18.1	18.1	18.1	18.2	18.3	18.4	18.8	19.3	5.0	4.4	–	68.6
15	42.7	42.8	42.9	42.6	42.6	42.6	42.4	42.4	42.8	42.7	43.4	40.8	40.3	40.8	–

Data in the upper triangle of the table are of sequence similarity (%), and the bottom triangle shows genetic distance

1, *Nosema* sp. MC; 2, *Nosema bombycis* CQ (EU350392); 3, *Nosema bombycis* GD (JF443586); 4, *Nosema heliothidis* (FJ772435); 5, *Nosema* sp.PX1 (AY960896); 6, *Nosema spodopterae* (AY211392); 7, *Nosema bombycis* (D85503); 8, *Nosema* sp.SC (FJ767862); 9, *Vairimorpha imperfecta* (AJ131646); 10, *Nosema antheraeae* (DQ073396); 11, *Nosema plutellae* (AY960897); 12, *Nosema ceranae* (DQ486028); 13, *Nosema vespula* (U11047); 14, *Nosema apis* (U97150) and 15, *Encephalitozoon cuniculi* (Z19563)

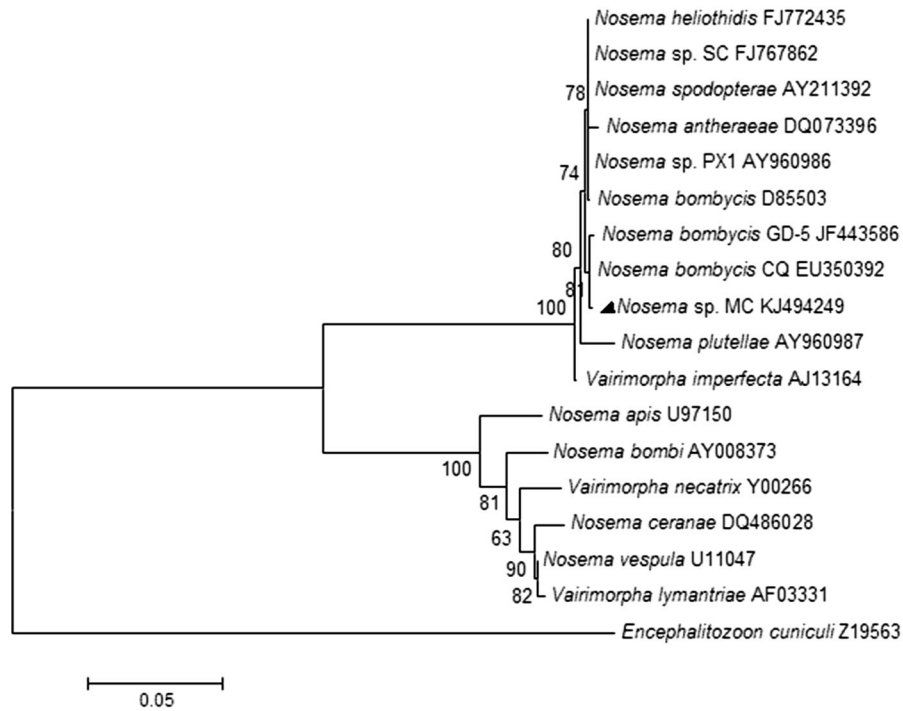

Fig. 4 Phylogenetic analysis of *Nosema* sp. MC based on SSU rRNA genes. Phylogenetic trees were constructed using the maximum likelihood (ML) method. The bootstrap values are labeled respectively

Molecular phylogenetic analysis can further validate a taxonomic status based on biological characteristics (Dong et al. 2010). SSU rRNA gene sequence comparison and phylogenetic analysis of *Nosema* sp. MC and other microsporidia showed that *Nosema* sp. MC fits the 'true' *Nosema* clade within the genus *Nosema* (Kyei-Poku et al. 2008). Furthermore, rRNA ITS gene sequences contained 181 base pairs and justly accorded with the length of interval of 'true' *Nosema* sub-group (Choi et al. 2011).

Based on the above, we confirmed that *Nosema* sp. MC belong to the genus *Nosema* and is a member of the sub-group of 'true' *Nosema*. Therefore, taking into account both biological characteristics and molecular data will leads to a more accurate description of microsporidian species.

Sequence alignment and phylogenetic analysis helped us to differentiate the related species and to understand the evolutionary relationships of the microsporidia (Zhu et al. 2011). The phylogenetic trees based on SSU rRNA sequences showed that *Nosema* sp. MC is most closely related to *N. bombycis* CQ-4 and *N. bombycis* GD-5. However, despite high sequence similarity, sequence alignments indicted that a nucleotide at position 824 is common in *N. bombi* (Accession Number: AY008373), *N. ceranae* (Accession Number: DQ486028), *V. necatrix* (Accession Number: Y00266) and *V. lymantriae* (Accession Number: AF03331) but rare in 'true' *Nosema* species. Furthermore, the rRNA ITS sequences of *Nosema* sp. MC differ substantially from *N. bombycis* CQ-4 (91.1 %) and *N. bombycis* GD-5 (92.5 %). These results indicated that *Nosema* sp. MC is a novel species and probably has an intimate genetic relationship with *N. bombycis* isolates from the Chongqing and Guangdong areas in China.

Cross-infection of the microsporidia from wild insects in silkworms has been reported. However, the idea did not arouse sufficient attention from the silkworm egg production units and farmers. Sanitation of the mulberry leaves has not been applied widely across the country. In this study, the pathogenicity of *Nosema* sp. MC to silkworm was studied. We found that the infectivity of *Nosema* sp. MC is comparable to *N. bombycis* and it also infects silkworms through vertical transmission from infected females to the offspring, and cause chromic deleterious effects on larvae. The incidence of transovarial transmission of *Nosema* sp. MC was nearly 60 %, which badly influenced the next generation of infected females. These results proved again that some microsporidia from wild insects have robust pathogenicity to silkworms and pose a grave threat to sericulture production. Thus, mulberry field administration should be strengthened to eliminate mulberry leave pollution by these organisms from wild insects (Takeshi 2003). Once the pathogenic microsporidium is identified, effective measures, such as sanitation of the mulberry leaves, should be carried out as early as possible.

This is the first formal description of a microsporidium from *M. cribraria*. Although attempts to rear microsporidia-infected *M. cribraria* for further study were unsuccessful, this finding was very interesting. Not many hemipteran microsporidia have been described and the behavior of the nymphs, which first feed on the feces of the female adult to obtain symbiotic gut microbes (Ezenwa et al. 2012), may suggest that the microsporidium uses a unique strategy for maintaining infection in the M. cribraria population. If so, it may become one of the pathogens that can be used in integrated pest management (IPM) of *M. cribraria* on crops (Kermani et al. 2013). More studies are still required in this regard.

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