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ORIGINAL PAPER

Ultrastructure, chromosomal karyotype, and molecular phylogeny of a new isolate of microsporidian *Vairimorpha* sp. BM (Microsporidia, Nosematidae) from *Bombyx mori* in China

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Abstract The spore morphology, chromosomal karyotype, and molecular systematic of a new microsporidian which was isolated from the domesticated silkworm *Bombyx mori* (Lepidoptera: Bombycidae) in Shandong, China have been studied. The spores were long oval and measured 3.4× 1.6 μm on fresh smears. Ultrastructure of the spores was characteristic for the genus *Vairimorpha*: 13–15 polar filament coils, posterior vacuole, and a diplokaryon. Six chromosome bands have been separated by pulsed field gel electrophoresis. The sequenced complete *rRNA* gene of this isolate is 4,231 bp long. Phylogenetic analysis based on *SSU rRNA* gene and *LSU rRNA* gene both revealed that this novel microsporidian which was isolated from *B. mori* had close relationship to the genus *Vairimorpha*, not to the genus *Nosema*. Moreover, the organization of the rRNA units of this microsporidian is not similar to that of *Nosema bombycis*, but same to that of other microsporidian, such as *Vairimorpha necatrix*. Although this microsporidian, designed as *Vairimorpha* sp. BM, was isolated from *B. mori*, all of these informations indicate that this isolate is closely related to the *Vairimorpha* group.

Nucleotide sequence reported in this study has been submitted to the GenBank™, EMBL, and DDBJ databases under the accession number HQ891818.

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Introduction

Microsporidia are minute (diameter, 1–40 μm), obligate intracellular parasites of other eukaryotes, ranging from protists to invertebrates and vertebrates, including human beings (Franzen and Muller 1999; Joseph et al. 2006). These organisms are emerging pathogens and are serious pests in sericulture, apiculture, and fisheries (Wittner and Weiss 1999). Of the 1,300 microsporidian species described in the literatures so far (Corradi et al. 2009), at least 200 belong to the genus *Nosema* (Sprague 1982). Moreover, the most common microsporidian that infect Lepidoptera are members of the genus *Nosema* (Tsai et al. 2003) 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).

Pébrine is an infectious disease, which usually caused by *Nosema bombycis*, and may destroy the development of sericulture, especially during breeding and cocoon production. In 2010, a novel microsporidian was isolated from the domesticated silkworm, *Bombyx mori* in Shandong, China and was reared in the laboratory. The microsporidian infections in the offspring of the silkworms were systemic and led to the collapse of the entire laboratory population. This isolate appeared to be morphologically different to that of the *N. bombycis*. A preliminary study revealed that the small subunit (SSU) rDNA and large subunit (LSU) rDNA of this isolate have high similarity to that of *Vairimorpha necatrix*. In this paper, we described this new isolate (designated as *Vairimorpha* sp. BM) based on its morphological characteristic, chromosomal karyotype, and its phylogenetic relationship with other microsporidian.

Materials and methods

Spore production and purification

Vairimorpha sp. BM was isolated from infected silkworms in Shandong, China. The third instar molted silkworm larvae were challenged by feeding on artificial diets contaminated by *Vairimorpha* sp. BM (about 1.0×10^5 spores for one silkworm, Liu et al. 2008). The silk gland of the infected larvae was dissected at day 3 or 4 of the fifth instar, and homogenized and centrifuged further. Spores were purified by a discontinuous Percoll density gradient (25%, 50%, 75%, and 100%, *v*/*v*) and centrifuged at 30,000×*g* for 40 min. 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 *Vairimorpha* sp. BM 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.

Pulsed field gel electrophoresis

The preparation of plugs for pulsed field gel electrophoresis (PFGE) analysis was performed as described before (Liu et al. 2008): purified spores were inserted in 1.4% low melting point agarose gel, then the solidified agarose plugs were incubated in solution I (EDTA, 0.1 M; SDS, 5%; and DTT, 0.1 M) at 50°C for 2 h and before treatment with solution II (EDTA, 0.5 M; SLS, 0.5%; and Proteinase K, 1.5 mg/mL) at 50°C for 48 h. The first PFGE program was run under the following conditions: 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid; and 1 mM EDTA), 12°C; 5 V/cm; and pulse time 60, 65, 70, 75, 80, and 90 s, with a total run time 60 h. And the second PFGE program was run under the following conditions: 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA); 10°C; 5 V/cm; and pulse time 80, 100, 150, 180, 240, and 300 s, with a total run time 72 h.

Molecular phylogenetic analysis

Genomic DNA was extracted as previously described (Liu et al. 2008). The primer sets used for *rRNA* gene amplification and the expected sizes of all amplicons are shown in Table 1. All primers are from the report of Huang et al. (2004), and ITSR primer is the invert complementary sequence to LS228F. The amplification was performed under the following conditions: after initial denaturation of DNA at 94°C for 5 min, 30 cycles were run; 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. Other sequences were obtained from the NCBI GenBank database and were aligned using the ClustalX 1.83 program. The *rRNA* gene sequence of *Encephalitozoon cuniculi* was used as out-group. Phylogenetic trees were reconstructed using the neighbor-joining method (Saitou and Nei 1987) implemented in MEGA 4.0 program (Tamura et al. 2007). Bootstrap support was evaluated based on 1,000 replicates.

Results

Morphological characteristics

Light microscopy revealed that fresh *Vairimorpha* sp. BM spores were generally long oval. They had a mean length and mean width of 3.4 μm (SD 0.3) and 1.6 μm (SD 0.2), respectively (Fig. 1a). Electron micrography of a longitudinal section of a mature spore revealed that the spore wall consisted of an electron-dense exospore (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 13–15 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. 1b). All the above-mentioned features correspond to the principle characteristics of the genus *Vairimorpha*.

Chromosomal karyotype of microsporidian *Vairimorpha* sp. BM

In order to get the chromosomal karyotype of *Vairimorpha* sp. BM, we use pulsed field gel electrophoresis to separate its chromosomes. At first, the *N. bombycis* karyotype as control, program of separating *N. bombycis* chromosomes was used to separate the chromosomes of *Vairimorpha* sp. BM. But, this program could not separate the clear chromosomal bands of *Vairimorpha* sp. BM (Fig. 2a).

Table 1 Primers used for amplification of *Vairimorpha* sp. BM rRNA

Primer	Sequence	Amplicon size (bp)
Small subunit rRNA		1,251
18f	5′-CACCAGGTTGATTCTGCC-3′	
1537r	5′-TTATGATCCTGCTAATGGTTC-3′	
Large subunit rRNA		2,110
LS228F	5′-GGAGGAAAAGAAACTAAC-3′	
ILSUR	5′-ACCTGTCTCACGACGGTCTAAAC-3′	
Internal transcribed spacer		662
HG4F	5′-GCGGCTTAATTTGACTCAAC-3′	
ITSR	5′-GTTAGTTTCTTTTCCTCC-3′	
Intergenic spacer		732
ILSUF	5′-TGGGTTTAGACCGTCGTGAG-3′	
5SR	5′-TACAGCACCCAACGTTCCCAAG-3′	

Also, from Fig. 2a, we can see its chromosomal bands may be larger than that of *N. bombycis*. So, the second program was used to separate large chromosomes. Using the secondary program, we found that the karyotype of *Vairimorpha* sp. BM was separated to six chromosomal bands (Fig. 2b). This means that this microsporidian is comprised of no less than six chromosomes. Compared with *N. bombycis*, the number of chromosomal bands of *Vairimorpha* sp. BM is fewer than that of *N. bombycis*, and the majority of its bands are more than 1,000 kb.

Analysis of the rRNA sequence of *Vairimorpha* sp. BM

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 *Vairimorpha* sp. BM *rRNA* gene was 4,231 bp long (GenBank accession no. is HQ891818). The gene arrangement from the 5′ end is as follows: the small subunit gene (*SSU rRNA*, 1,251 bp), internal transcribed spacer (*ITS*, 29 bp), *5.8S rRNA* gene (*5.8S*, 93 bp), large subunit gene (*LSU rRNA*, 2,462 bp), intergenic spacer (*IGS*, 277 bp), and *5S rRNA* gene (5S, 119 bp). The sequence identity of the *Vairimorpha* sp. BM *rRNA* to the *rRNA* of other sequenced microsporidian species has been compared. The *LSU* and *SSU* regions of the *Vairimorpha* sp. BM *rRNA* show 96–99% identity with the corresponding regions in the *rRNA* of *Vairimorpha* species (*V. necatrix*, *Vairimorpha* sp. *NIS-M12*, *Vairimorpha* sp., *Vairimorpha* sp. *C21*, and *Vairimorpha lymantriae*). The results also suggest that this isolate may be most closely related to *V. necatrix*, with which it shares 99% identity of total *rRNA* gene (LSU, 99%; ITS, 99%; 5.8S, 98%; and SSU, 99%). The *rRNA* of the novel isolate also shares high sequence similarity with that of the uncultured *Nosema* clone MPr (GenBank accession no. HQ399665): 96% identity of the total *rRNA* gene.

Molecular phylogeny

The *SSU rRNA* gene of *Vairimorpha* sp. BM consists of 1,251 bp nucleotides, and the GC content is 37.33%. Based on SSU rRNA sequences, two genera, *Vairimorpha* and *Nosema*, formed a complex (Fig. 3a). From the phylogenetic tree, we can see that *Vairimorpha* sp. BM is unique and shares the same ancestor with other species within the *Vairimorpha* complex. The identities of *SSU rDNA* sequences between

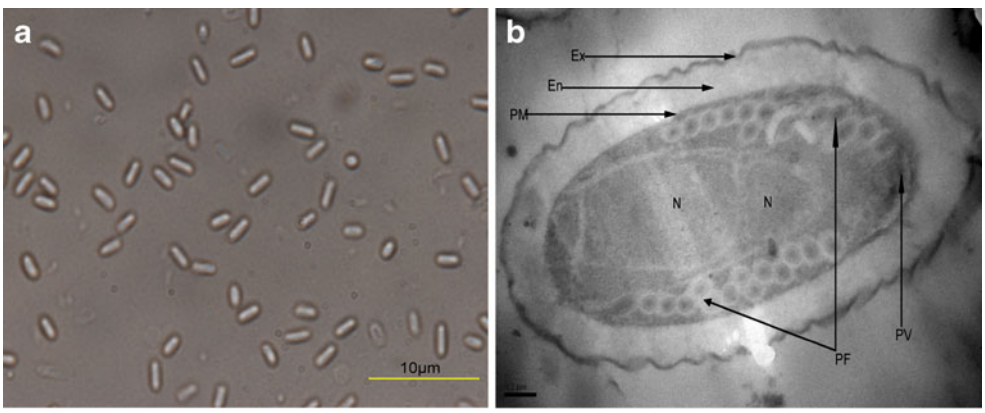

Fig. 1 Micrographs of the *Vairimorpha* sp. BM spores. **a** Light micrograph of the *Vairimorpha* sp. BM spores after Percoll purification. *Scale bar*=10 μm. **b** Electron micrograph of a longitudinal section of a *Vairimorpha* sp. BM spore. The nucleus (*N*), exospore (*Ex*), endospore (*En*), plasma membrane (*PM*), polar filament (*PF*), and posterior vacuole (*PV*) are visible. *Scale bar*=0.2 μm

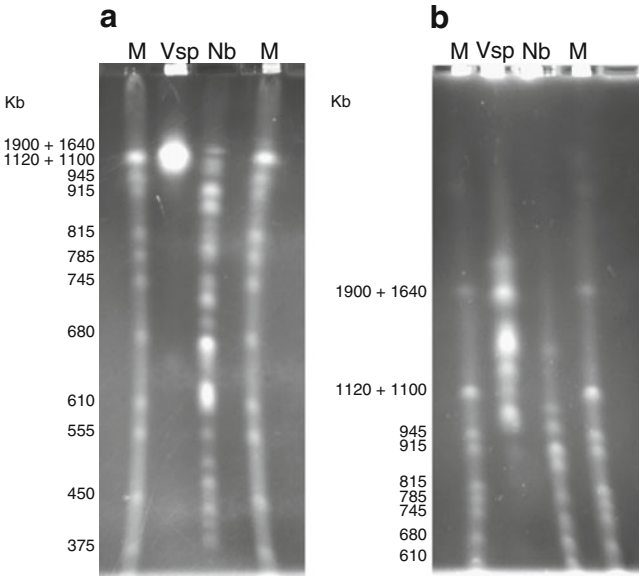

Fig. 2 Electrophorogram segregated by PFGE of *Vairimorpha* sp. BM. **a** PFGE is performed by the first program. **b** PFGE is performed by the second program. *M*: yeast chromosome PFG marker; *Vsp*: *Vairimorpha sp*. BM; *Nb*: *Nosema bombycis*

Vairimorpha sp. BM and other species within the *Vairimorpha* complex are 96–99%. Identity between *Vairimorpha* sp. BM and the type species of the genus *V. necatrix* is 99%. The identities between *Vairimorpha* sp. BM and *Nosema* species within *Vairimorpha* complex are 97–99%, but only are 84–86% between *Vairimorpha* sp. BM and species within the "true" *Nosema* complex (type species *N. bombycis*).

The sequenced *LSU rDNA* sequence of *Vairimorpha* sp. BM consists of 2,462 bp nucleotides, and the GC content is 32.74%. The phylogenetic tree of *LSU rDNA* sequences is shown in Fig. 3b. Among the sequences in this tree, the identities of *LSU rDNA* sequences between *Vairimorpha* sp. BM and other species within the *Vairimorpha* complex are 98%. The identities between *Vairimorpha* sp. BM and *Nosema* species within *Vairimorpha* complex are 90–93%, but only 79–80% between *Vairimorpha* sp. BM and species within the "true" *Nosema* complex (type species *N. bombycis*). All *Vairimorpha* species analyzed are parasites of lepidopteran insects.

Symptom of silkworm infected by *Vairimorpha* sp. BM

Using the same method of feeding *N. bombycis* to silkworm, we feed the *Vairimorpha* sp. BM spores to the

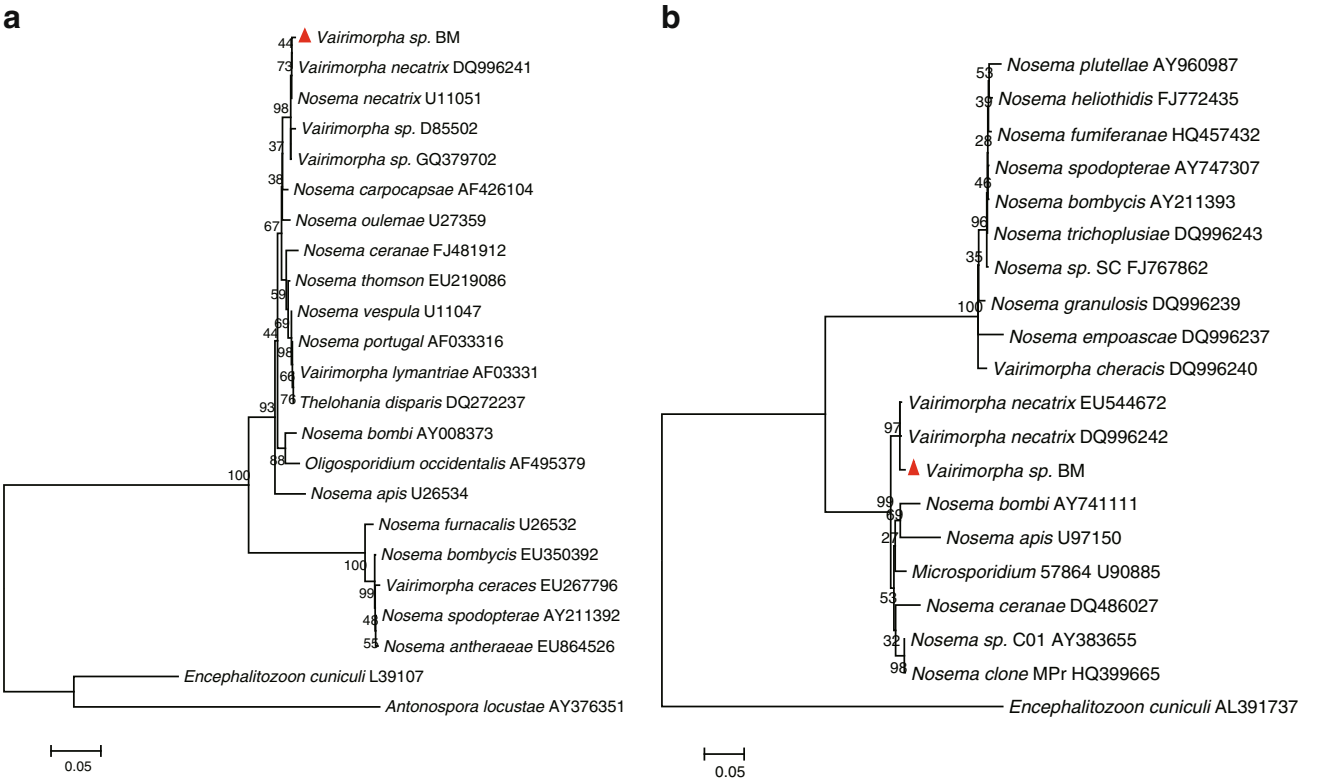

Fig. 3 Phylogenetic analysis of the *Vairimorpha* sp. BM based on the *SSU rRNA* and *LSU rRNA* genes. **a** A phylogenetic tree based on the SSU rRNA sequences. **b** A phylogenetic tree based on the LSU rRNA sequences. Phylogenetic trees were constructed by using the neighbor-joining method. The bootstrap values are indicated at the nodes. The GenBank accession number for each sequence is given adjacent to the corresponding species name

silkworm *B. mori* (Dazao). There are dead larvae in the infected silkworms after 8 days by feeding (Fig. 4). This result is consistent to previous report that when the third instar larvae of *Lacanobia oleracea* ingested a dose of 200,000 spores of *V. necatrix*, there was 80% mortality within 6 days(Down et al. 2004). The body wall of infected silkworms (Fig. 4b, d) is more compact than that of the control silkworms (Fig. 4a, c). And the development of infected silkworms is abnormal (such as the instar period of infected silkworms is longer than that of the normal one) compared with the control silkworms. Furthermore, all infected silkworms are dead after 10 days from being reared by *Vairimorpha* sp. BM spores.

Discussion

There are lots of reports about microsporidian after the first microsporidian *N. bombycis* which had been documented in 1857. Now, we obtain a new isolate of microsporidian, *Vairimorpha* sp. BM, from the domesticated silkworm *B. mori* in Shandong, China. As we all know, the *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. Therefore, other markers were required for analyzing phylogenetic affinities. Tsai et al. (2005) suggested that the microsporidian *ITS* and *LSU rRNA* sequences have good potential as informative molecular markers. According to the results of this study, the identity of the *rRNA* gene sequences of *Vairimorpha* sp. BM with those of the other *Vairimorpha* species is high. Phylogenetic analysis of *SSU rRNA* and *LSU rRNA* (Fig. 3a, b) revealed that this isolate is closely related to the members of the *Vairimorpha* group.

According to the PFGE result, there are six chromosomal bands that can be found. This number is less than the chromosome numbers of *N. bombycis* (Kawakami et al. 1994; Liu et al. 2008). And this result agrees to the reports that one *Vairimorpha* isolate from *Pieris rapae* has a minimum of eight bands and a *Vairimorpha* sp. has fewer than 13 chromosomes (Munderloh et al. 1990; Malone and McIvor 1993). For the exact number of *Vairimorpha* sp. BM, there is still a need for some experiments to be done in the future.

For this microsporidian isolated from *B. mori*, *Vairimorpha* sp. BM not only has more similarity in sequence to *V. necatrix* than to *N. bombycis*, but also the organization of the *rRNA* gene of *Vairimorpha* sp. BM is 5′-SSU-ITS-LSU-IGS-5S-3′, which is a pattern different to the *rRNA* gene of *N. bombycis* (Huang et al. 2004), *Nosema spodopterae* (Tsai et al. 2005), *Nosema antheraeae* (Wang et al. 2006), *Nosema plutellae* (Ku et al. 2007), and uncultured *Nosema* (Tsai et al. 2009). This *rRNA* gene arrangement of *Vairimorpha* sp. BM may be an important difference to that of the "true" *Nosema* group.

Fig. 4 Normal and infected silkworm (Dazao) by *Vairimorpha* sp. BM. **a**, **c** The normal silkworms. **b**, **d** The silkworms infected by *Vairimorpha* sp. BM

According to the results of the morphological, genetic characteristics, and chromosome karyotype analyses, we suggest that the microsporidian *Vairimorpha* sp. BM belongs to the *Vairimorpha* group. So, we also can declare that not only *N. bombycis* can result in loss in sericulture, but also the other microsporidian (such as *Vairimorpha* sp. BM) can.

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