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ORIGINAL PAPER

# Morphological and phylogenetic analysis of a microsporidium (*Nosema* sp.) isolated from rice stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae)

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**Abstract** A new microsporidium was isolated from *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae), one of the most important rice pests in China. The morphology and molecular systematics of this novel microsporidium were described in this study. The spores were long oval and measured 3.17 × 1.64 μm on fresh smears. Ultrastructure of the spores was characteristic for the genus *Nosema*: a diplokaryon, 10–12 polar filament coils of the same type, and posterior vacuole. Small subunit rRNA gene sequence data and phylogenetic analysis further confirmed that the microsporidian species from *C. suppressalis* belong to the true *Nosema* sub-group of the genus *Nosema*. Besides, the microsporidium *Nosema* sp. CS could cause systemic infection of *Bombyx mori* and infect silkworms through vertical transmission. Therefore, mulberry field pest control should be carefully monitored, and sanitation of mulberry leaves is essential to control the pebrine disease in sericulture.

**Keywords** Microsporidium · *Nosema* sp. CS · SSU rRNA · Morphology · Phylogenetic analysis

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## Introduction

Microsporidia are unicellular, obligate intracellular parasites that can infect nearly all vertebrate and invertebrate, including human beings (Keeling 2009; Joseph et al. 2006; Morsy et al. 2013; Ovcharenko et al. 2017). More than 1500 microsporidial species belonging to 187 genera have been formally described so far (Wittner and Weiss 1999; Xu et al. 2016). The genus *Nosema* is the most diverse, containing at least 200 species. *Nosema bombycis*, the type species of the genus *Nosema*, causes pebrine disease in sericulture causing devastating economic losses (Pan et al. 2013).

In addition to *N. bombycis*, some microsporidia from wild insects (especially Lepidoptera) could also infect the silkworms by the polluted mulberry leaves (Yang et al. 2007; Huang et al. 2011). The microsporidia from wild insects is becoming an important factor of causing pebrine disease in silkworms (Liao et al. 2016). Thus, the accurate identification of those unknown microsporidia has aroused sufficient attention and may play an important role in prognosis and prevention assessment of the pebrine disease (Huang et al. 2013).

The rice stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae), is one of the most important rice pests in China, posing a serious threat to the high and stable yields of the crop (He et al. 2008; Wu et al. 2014). We isolated a novel microsporidium from the rice stem borer, *Chilo suppressalis* (Walker). It not only infected *C. suppressalis* heavily but also caused systemic infection of *Bombyx mori* larvae. The isolate was shown to be morphologically and genetically similar to the species of the *N. bombycis* group. Here, we described the parasite (designated as *Nosema* sp. CS) based on its morphological characteristics and phylogenetic relationships with other microsporidia.

## Materials and methods

### Host insect and spore purification

The larvae of *C. suppressalis* infected by microsporidian were provided by Dr. Han Lanzhi from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences. The infected larvae were homogenized, filtered through three layers of cheesecloth, and centrifuged at 3000×*g* for 15 min (Xing et al. 2014). Then, the pellets were resuspended in sterile water and further purification was carried out as described previously (Tsai et al. 2009).

### Light and scanning electron microscopy

The purified spores were observed under a light microscope (OLYMPUS CX43) with an ocular micrometer and photographed with the microscope USB camera. For scanning electron microscopy (SEM), the spores were centrifuged and the pellets were fixed in 2.5% (*v*/*v*) glutaraldehyde buffer in 0.1 M phosphate buffer (pH 7.2), rinsed with 0.1 M phosphate buffer, fixed in 1% osmic acid (*w*/*v*) for 2 h, rinsed with 0.1 M phosphate buffer, and dehydrated in ascending alcohol series. The spores were then subjected to isoamyl acetate replacement, once in 50% and twice in 100% isoamyl acetate. An XD-1 carbondioxide critical point dryer (Eiko, Japan) was used for drying, and a JFC 1600 ion sputter coater (Jeol, Japan) was used for spray gold plating. An AJSM-6360 LV scanning electron microscope (Jeol, Japan) was used for observation.

### Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as previously described (Xing et al. 2011). The purified spores of *Nosema* sp. CS were fixed in 6% (*v*/*v*) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight. The spores were centrifuged and the pellets were encapsulated with 1.5% agarose solution. The embedded samples were rinsed in 0.1 M cacodylate buffer, fixed in 1% osmic acid (*w*/*v*) for 2 h, and rinsed again in cacodylate buffer. Then, the samples were postfixed for 1 h with 0.5% potassium permanganate (*w*/*v*), dehydrated through an ascending ethanol series, and 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 G2 12 electron microscope with an accelerating voltage of 80 kV.

### Genomic DNA extraction and PCR amplification

Genomic DNA was extracted using MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Shiga, Japan). The primer 18f/1537r was used to amplify small subunit (SSU) ribosomal RNA (rRNA) gene sequence, and ILSUF/S33R was used to amplify rRNA internal transcribed spacer (ITS) gene sequence (Dong et al. 2010; Zhu et al. 2011). Polymerase chain reactions (PCRs) were performed with an Applied Biosystems 2720 Thermal Cycler PCR (Thermo Fisher Scientific, Waltham, MA, USA) in a 25-μL reaction volume. The PCR program was as follows: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, annealing temperature for 60 s, and elongation at 72 °C for 90 s. The PCR products were purified using MiniBEST Universal DNA Fragment Purification Kit (TaKaRa) and cloned into pMD18-T vector and sequenced by Beijing Liuhe Huada Genomics Technology Co., Ltd.

### Molecular phylogenetic analysis

The SSU rRNA sequence of *Nosema* sp. CS and other microsporidia sequences obtained from the NCBI GenBank database (Table 1) were aligned using the Clustal X 1.83 program (Thompson et al. 1997). The SSU rRNA sequence of *Encephalitozoon cuniculi* was used as the outgroup. Phylogenetic tree based on SSU rRNA gene sequences was constructed using the maximum likelihood method with MEGA 6 program (Tamura et al. 2013). One thousand bootstrap replicates were used to test the robustness of the trees.

### Pathogenicity to the silkworms

Second instar larvae of the silkworms were inoculated per os with $1 \times 10^6$ spores $ml^{-1}$ of *Nosema* sp. CS. Ten days later, the larvae were dissected and the tissues including midguts, silkglands, gonads, malpighian tubules, and muscle and fat bodies were identified individually. Each sample was washed twice in physiological saline and observed by phase contrast microscopy. Also, transovarial transmission of *Nosema* sp. CS was tested using the method as previously described by Xing et al. (2014).

## Results

### Spore morphology and size

Light microscopy revealed that fresh spores were highly refractive, typical of microsporidia (Fig. 1a). The spores showed clear differences from *N. bombycis* in shape and size (Table 2). They were long oval and measured 3.17 ± 0.25 μm long by

**Table 1** NCBI GenBank accession numbers for of *Nosema* sp. CS and other species isolated from insects used for phylogenetic analysis

| Microsporidia | Host | GenBank accession no. |
|---|---|---|
| *Nosema* sp. CS | *Chilo suppressalis* | MF094454 |
| *Nosema* sp. PM-1 | *Papilio machaon* | KM190863 |
| *Nosema spodopterae* | *Spodoptera litura* | AY747307 |
| *Nosema heliothidis* | *Helicoverpa armigera* | FJ772435 |
| *Nosema bombycis* | *Bombyx mori* | AY259631 |
| *Nosema* sp. MC | *Megacopta cribraria* | KJ494249 |
| *Nosema plutellae* | *Plutella xylostea* | AY960987 |
| *Nosema furnacalis* | *Ostrinia furnacalis* | U26532 |
| *Nosema vespula* | *Helicoverpa armigera* | U11047 |
| *Vairimorpha lymantriae* | *Lymantria dispar* | AF033315 |
| *Vairimorpha necatrix* | *Pseudaletia unipuncta* | Y00266 |
| *Nosema bombi* | *Bombus* spp. | AY008373 |
| *Nosema ceranae* | *Apis cerana* | DQ486028 |
| *Nosema apis* | *Apis mellifera* | U97150 |
| *Encephalitozoon cuniculi* | Mammal | Z19563 |

1.64 ± 0.12 μm wide ($n$ = 30). SEM showed that the spores were smooth, without any wrinkles or protrusions (Fig. 1b).

## Spore ultrastructure

A longitudinal TEM section of the mature spore revealed that the spore wall 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 was found in the anterior end of the spore and connected with the straight anterior region of the polar filament. The polar filament extended laterally over the polaroplast and gradually formed coils. The isofilar polar filament was arranged in 10–12 coils and always occurred in a single rank. The diplokaryotic nuclei were slightly separated from each other. The polaroplast consisted of an anterior region of closely packed membranes and a posterior region comprised a series of loosely packed membranes. A membrane-bound vacuole with amorphous content was

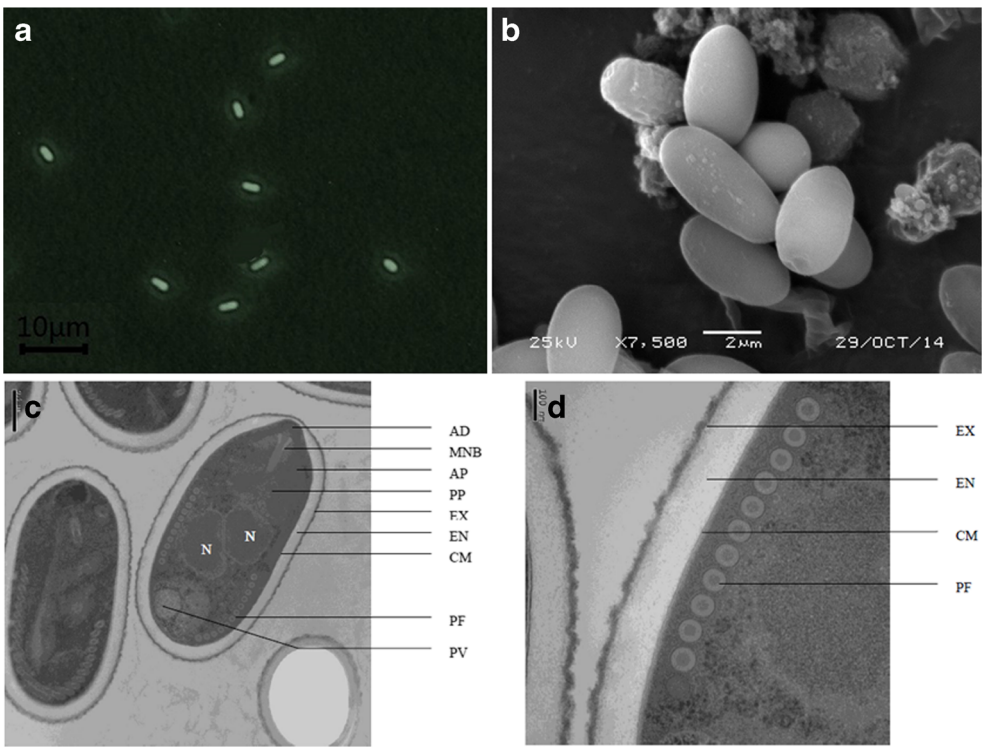


**Fig. 1** Micrographs of the *Nosema* sp. CS spores. **a** Mature spores under light microscope. Scale bar = 10 μm. **b** Surface structure of spores under scanning electron microscopy (SEM). Scale bar = 2 μm. **c** Electron micrograph of a longitudinal section of *Nosema* sp. CS spore. The anchoring disk (AD), manubraid (MNB), anterior polaroplast (AP), posterior polaroplast (PP), exospore (EX), endospore (EN), cytoplasm membrane (CM), nucleus (N), polar filament (PF), and posterior vacuole (PV) are visible. Scale bar = 0.5 μm. **d** 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

**Table 2** The shape and size of *Nosema* sp. CS and *N. bombycis* spores

| Microsporidia | Shape | Long axis (μm) | Short axis (μm) | *P* value of long axis | *P* value of short axis |
|---|---|---|---|---|---|
| *Nosema* sp. CS | Long oval | 3.17 ± 0.25 | 1.64 ± 0.12 | 0.028* | 0.004** |
| *N. bombycis* | Oval | 3.73 ± 0.41 | 1.96 ± 0.24 | | |

*Represented significant difference at $P < 0.05$

**Represented significant difference at $P < 0.01$

located at the posterior end of the spore and occupied approximately one-third of the posterior region (Fig. 1c, d). All the features corresponded to the principle characteristics of the genus *Nosema* (Larsson 1988).

## Phylogenetic analysis

The SSU rRNA gene sequence of *Nosema* sp. CS was deposited at the GenBank database (accession number: MF094454). The amplified SSU rRNA gene sequence was 1231 bp long, with ~ 34.28% G + C content. The similarity and genetic divergence of the SSU rRNA gene sequence of the microsporidium and other 14 microsporidian species are shown in Table 3. It exhibited higher identities with true *Nosema* species (97.9–99.7%), while lower identities with non-true *Nosema* species (82.3–84.0%). The results indicated that it may be most closely related to *Nosema* sp. PM-1, *Nosema spodopterae*, and *Nosema heliothidis*, with which they shared 99.7% identities. In addition, it also had 99.6% sequence identities with *N. bombycis*.

Furthermore, the maximum likelihood analysis based on SSU rRNA sequences was performed (Fig. 2). Data from the phylogenetic tree of SSU rRNA sequence revealed that *Nosema* sp. CS was unique and shared the same ancestor with other species within true *Nosema* complex. It was found to be most closely related to *Nosema* sp. PM-1, *N. spodopterae*, and *N. heliothidis* followed by *N. Bombycis*, which was coincident with that of SSU rRNA sequence analysis.

The rRNA ITS fragment was also obtained by PCR amplification (accession number: MF098550). It contained 181 bp with ~ 20.44% G + C content and justly accorded with the length of interval of true *Nosema* sub-group (Choi et al. 2011). NCBI-BLAST analysis revealed that the isolate was most closely related to *N. bombycis* YN-4 (accession number: JF443650) and exhibited 96.1% sequence identity.

**Table 3** The similarity and genetic divergence of SSU rDNA gene sequences among 15 microsporidian species

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | – | 99.7 | 99.7 | 99.7 | 99.6 | 99.4 | 98.3 | 97.9 | 84.0 | 83.8 | 83.5 | 82.7 | 82.6 | 82.3 | 61.0 |
| 2 | 0.1 | – | 100.0 | 100.0 | 99.9 | 99.7 | 98.6 | 98.1 | 84.3 | 84.0 | 83.7 | 82.9 | 82.9 | 82.5 | 61.2 |
| 3 | 0.1 | 0.0 | – | 100.0 | 99.9 | 99.7 | 98.6 | 98.1 | 84.3 | 84.0 | 83.7 | 82.9 | 82.9 | 82.5 | 61.2 |
| 4 | 0.1 | 0.0 | 0.0 | – | 99.9 | 99.7 | 98.6 | 98.1 | 84.3 | 84.0 | 83.7 | 82.9 | 82.9 | 82.5 | 61.2 |
| 5 | 0.2 | 0.1 | 0.1 | 0.1 | – | 99.8 | 98.7 | 98.2 | 84.3 | 84.3 | 83.7 | 82.9 | 82.9 | 82.5 | 61.1 |
| 6 | 0.4 | 0.3 | 0.3 | 0.3 | 0.2 | – | 98.5 | 98.1 | 84.3 | 84.3 | 83.8 | 83.3 | 82.9 | 82.6 | 61.0 |
| 7 | 1.6 | 1.5 | 1.5 | 1.5 | 1.4 | 1.6 | – | 96.8 | 83.4 | 83.4 | 82.6 | 81.7 | 82.0 | 82.1 | 60.6 |
| 8 | 2.0 | 2.0 | 20 | 2.0 | 1.9 | 2.0 | 3.3 | – | 84.2 | 84.2 | 84.4 | 82.8 | 82.8 | 83.1 | 61.2 |
| 9 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 17.6 | 16.2 | – | 99.8 | 96.7 | 96.6 | 97.1 | 94.3 | 62.6 |
| 10 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 16.5 | 17.6 | 16.2 | 0.2 | – | 96.5 | 96.7 | 96.9 | 94.1 | 62.3 |
| 11 | 16.6 | 16.6 | 16.6 | 16.6 | 16.6 | 16.4 | 17.7 | 16.0 | 2.6 | 2.9 | – | 94.9 | 95.1 | 93.4 | 62.6 |
| 12 | 17.2 | 17.1 | 17.1 | 17.1 | 17.1 | 16.9 | 18.3 | 16.9 | 2.6 | 2.5 | 3.7 | – | 94.5 | 94.6 | 63.5 |
| 13 | 17.5 | 17.5 | 17.5 | 17.5 | 17.5 | 17.4 | 18.7 | 17.1 | 1.5 | 1.8 | 3.6 | 3.6 | – | 92.5 | 62.2 |
| 14 | 17.5 | 17.5 | 17.5 | 17.5 | 17.5 | 17.4 | 18.4 | 17.3 | 4.7 | 4.9 | 4.9 | 5.4 | 5.3 | – | 63.4 |
| 15 | 44.2 | 44.0 | 44.0 | 44.0 | 44.1 | 44.1 | 44.8 | 44.0 | 40.6 | 40.7 | 40.2 | 38.9 | 41.6 | 40.2 | – |

Data in the upper triangle of the table are of sequence similarity (%), and the bottom triangle shows genetic divergence. 1, *Nosema* sp. CS (MF094454); 2, *Nosema* sp. PM-1 (KM190863); 3, *Nosema spodopterae* (AY747307); 4, *Nosema heliothidis* (FJ772435); 5, *Nosema bombycis* (AY259631); 6, *Nosema* sp. MC (KJ94249); 7, *Nosema plutellae* (AY960987); 8, *Nosema furnacalis* (U26532); 9, *Nosema vespula* (U11047); 10, *Vairimorpha lymantriae* (AF033315); 11, *Vairimorpha necatrix* (Y00266); 12, *Nosema bombi* (AY008373); 13, *Nosema ceranae* (DQ486028); 14, *Nosema apis* (U97150); and 15, *Encephalitozoon cuniculi* (Z19563)

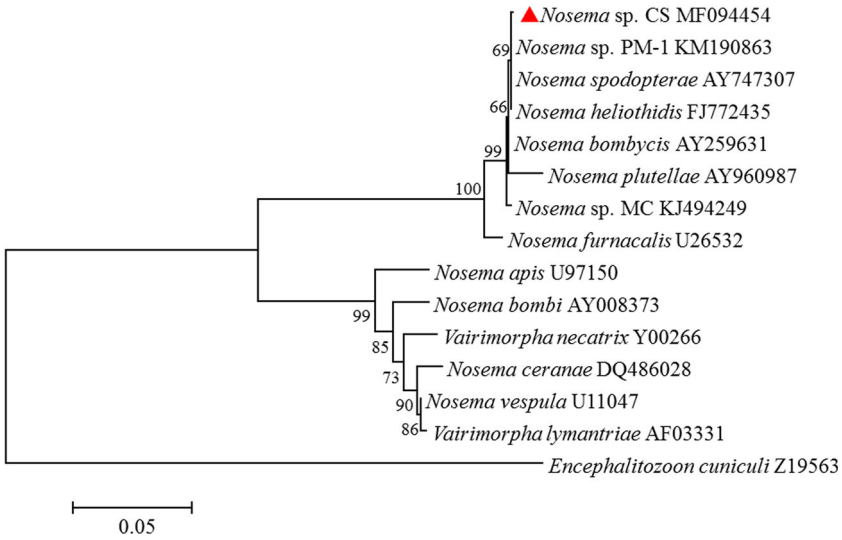


**Fig. 2** Phylogenetic analysis of *Nosema* sp. CS based on SSU rRNA gene. Phylogenetic trees were constructed by the maximum likelihood method. The bootstrap values are indicated at the nodes. The GenBank accession number for each sequence is given adjacent to the corresponding species name. The *Nosema* sp. CS sequence has been indicated by red triangle in the phylogenetic tree

On the basis of both analyses, the *C. suppressalis* isolate was identified as a new species of the "true" *Nosema* group of the genus *Nosema*.

### Pathogenicity of *Nosema* sp. CS to silkworms

Tissue tropism of *Nosema* sp. CS was determined in the fifth instar larvae of *B. mori*. The tissues of midguts, gonads, malpighian tubules, fat body, and silk glands were all infected, indicating that the parasites cause a systemic infection to the silkworms. In addition, tests for transovarial transmission showed that *Nosema* sp. CS could infect silkworms through vertical transmission from the mother host to their progenitive eggs.

## Discussion

The traditional taxonomy of microsporidia has long been established on the basis of spore morphological characteristics, including the shape, size, and ultrastructure, as well as lifecycle stages and host range (Larsson 1988; Sprague et al. 1992). In our study, the spores were long oval and measured $3.17 \pm 0.25 \times 1.64 \pm 0.12$ μm, which was significantly different from *N. bombycis* in shape. Besides, TEM observation indicated that the mature spore is dikaryotic and has about 10–12 polar filament coils. The polar filament coils were of nearly identical diameter and each coil was composed of five layers of alternately dark and bright materials. Additionally, *Nosema* sp. CS had transovarial transmission and systemic infection to type species of lepidopteran hosts, *B. mori*. Therefore, the biological characteristics of *Nosema* sp. CS corresponded to the genus *Nosema* (Iwano and Ishihara 1991; Didier et al. 2004).

Gene sequences, especially rRNA gene sequences of microsporidia, have been increasingly used in the classification of microsporidia (Wittner and Weiss 1999; Huang et al. 2004; Ku et al. 2007; Ma et al. 2008; Zhu et al. 2010). By means of phylogenetic analysis based on rRNA sequences, the genus *Nosema* was divided into two sub-groups: the true *Nosema* group (type species *N. bombycis*) and the non-true *Nosema* group consisting of species of *Vairimorpha* (type species *Vairimorpha necatrix*) and some other *Nosema* spp. (Baker et al. 1994; Liu et al. 2012). Phylogenetic analysis has been believed to help amend a taxonomic status based on biological characteristics. Based on the phylogenetic analysis of SSU rRNA sequences, *Vairimorpha imperfecta* and the German isolate of *Vairimorpha* from the host *Plutella xylostella* were found to be true *Nosema* species rather than *Vairimorpha* species (Canning et al. 1999). In this study, SSU rRNA gene sequence analyses and phylogenetic tree of *Nosema* sp. CS revealed that the isolate is a novel species of the true *Nosema* group and was found to be most related to *Nosema* sp. PM-1, *N. spodopterae* and *N. heliothidis*. Thus, the biological characteristics and molecular data are both valuable to elucidate phylogenetic relationships and taxonomic status of microsporidian species (Huang et al. 2013).

According to the results of the morphological and phylogenetic characteristics of *Nosema* sp. CS, we suggested that the novel microsporidium *Nosema* sp. CS belongs to the true *Nosema* group. Like most microsporidia from wild lepidopteran insects, *Nosema* sp. CS could also infect *B. mori* per os and transovarially. Therefore, silkworm egg production units should be especially attentive to control the mulberry field pests (Liu et al. 2014). In addition, sanitation of the mulberry leaves is essential to control the pebrine disease.

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