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Isolation and identification of *Cordyceps cateniobliqua* Bm1 and its pathogenicity on the silkworm, *Bombyx mori*

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ABSTRACT: Silkworm's fungal infection is a kind of communicable diseases caused by parasitic fungi. In this study, a rare fungal Bm1 strain was isolated from infected silkworms in cocoon production in Zhenjiang city, China. The result from morphological investigation showed that the conidiogenous cells of the isolated fungal strain had flask-like phialides with slightly swollen or columnar bases. Most of the conidia were oval and arranged in a broadly 'V'-like manner that resulted in irregularly curved chains. The color of the fungal colony was white at the initial growing stage and gradually became pink. The average hyphal growth rate of the strain was 3.42 ± 1.24 mm/d on the PDA solid medium. Phylogenetic analysis showed that the Bm1 strain was clustered in the same clade with *Isaria cateniobliqua*, *Cordyceps cateniobliqua*, and *Evlachovaea kintrischica*. Combining the morphological characteristics with rRNA-ITS sequence, this newly isolated fungal strain was identified as a *C. cateniobliqua* strain, named as C. *cateniobliqua* Bm1. The *C. cateniobliqua* Bm1 could infect the silkworm through contacting transmission, and the course of this disease was about 5 to 19 d. The mortality rates of the silkworm caused by infection of *C. cateniobliqua* Bm1 positively increased with the concentration of inoculated conidial suspension. The estimated LC_{50} value was 1.18×10^9 conidia/ml, a low pathogenicity to the silkworm compared with the *Beauveria bassiana*.

KEYWORDS: *Cordyceps cateniobliqua*, morphological analysis, pathogenicity, rRNA-ITS sequence, silkworm

INTRODUCTION

As a model organism of the lepidopteran insects, the silkworm can be used as the research objects in medicine and the control of lepidopteran pests [1]. The fungi are one kind of pathogens that can cause silkworm diseases. There are many kinds of silkworm fungal diseases that were caused by different entomopathogenic fungi such as *Beauveria bassiana*, *Metarhizium rileyi*; and the diseases were called white muscardine, green muscardine, and so on, according to the color of conidial colony on the body surface of the dead silkworm. The conidia of fungal pathogens attach on the surface of silkworms through air, mulberry leaves, and tools. Then, the attached conidia germinate and penetrate the cuticle under an appropriate condition, followed by the fungal growth and proliferation in the hemocoel of the silkworm [2].

The *Isaria* genus was established by basing on *I. terrestris* Fr. in the 19th century; however, most species of *Isaria* genus were classified in the genus *Paecilomyces* in the 20th century [3]. Combining the result of morphological technique with the phylogenetic analyses based on the ITS and β-tubulin sequences, Hodge thought that the *Isaria* was an effective generic name; so, the *Isaria* was reclassified as a genus in 2005 [4, 5]. Recently (in 2017), the *Isaria* and the *Evlachovaea* were classified in the genus *Cordyceps* [6]. The entomopathogenic fungus genus *Cordyceps* includes many species, such as *C. amoenerosea*, *C. cateniobliqua*, *C. cateniannulata*, *C. farinose*, *C. fumosorosea*, and *C. javanica*. The *Cordyceps* on the medium is displayed in bright colours: yellow, red, white, green, etc [7, 8]. *C. farinose* has highly insecticidal activity and can infect *Myzus persicae*, *Apocheima cinerarius* Ershoff, and larvae of *Cnidocampa flavescens*. *C. fumosorosea* can parasitize many pest insects, such as *Bemisia argentifolii* Bellow & Perring and *Plutella xylostella*. Up to now, three *Cordyceps* fungi, *C. farinose*, *C. fumosorosea* and *C. javanica* were reported to respectively cause the silkworm infections of yellow muscardine, red muscardine, and grey muscardine [9]. In this study, one rare fungal strain was isolated from infected silkworms; and through the morphological and ITS sequence analysis, the strain was identified as *C. cateniobliqua* Bm1. The morphology of this newly isolated fungal strain and the growth of its vegetative hypha were recorded. In addition, the pathogenicity of the strain to silkworms was analyzed.

MATERIALS AND METHODS

Silkworms and the fungal strain

The fungal *C. cateniobliqua* Bm1 (Bm1) strain was isolated from infected silkworms and stored on the PDA inclined medium at 4 °C. The silkworm race used

in this study was "Jingsong Haoyue".

Medium

Potato dextrose agar (PDA) medium, prepared by conventional method, contained 3% (w/v) fresh peeled potatoes, 2% (w/v) glucose, and 2% agar. Czapek-Dox medium was prepared by dissolving chemicals ($NaNO_3$ 2 g, KCl 0.5 g, $Fe_2(SO_4)_3$ 0.02 g, $MgSO_4$ 0.5 g, sucrose 30 g, and $K_2HPO_4 \cdot 3H_2O$ 1.31 g) in sterilized water, then made to a final volume of 1 l.

Collection and isolation of the fungal strain

Conidia of the strain were collected from the surface of the dead silkworm and inoculated on the PDA solid medium. After cultured at 25 °C for 7 days, fungal colonies showing the same colour and shape were transferred to a new PDA solid medium at 25 °C to be cultured for 10 days. Then, the conidia were collected and inoculated on the PDA inclined medium; the isolated fungal strain was stored at 4 °C.

Morphological observation and growth detection of vegetative hypha

After the 10 day culture at 25 °C on the PDA solid medium, a fungal colony with a diameter of 5 mm was taken with a hole-puncher to a petri dish and, then, inoculated on a new PDA medium and cultured at 25 °C. The diameter of the growing fungal colony was measured every day for 10 days from the day after the inoculation in 3 repetitive groups. The growth of vegetative hypha and the change of colony morphology and colour on the PDA solid medium were recorded. Additionally, the growth of the fungal strain was observed on Czapek-Dox medium. The morphology of conidiogenous cells and conidia were observed under a light microscopy.

Gene cloning and phylogenetic analysis of the fungal isolate

After the 10 day culture at 25 °C on the PDA solid medium, about 20 mg hyphae was collected from the medium and milled by liquid nitrogen, then, the powder was put into a 1.5 ml centrifuge tube. The genomic DNA of the strain was extracted using Rapid Fungi Genomic DNA Isolation Kit (Sangon Biotech (Shanghai) Co., Ltd), and the DNA concentration was determined. Then, the fungal genomic DNA was stored at −20 °C. rRNA-ITS was amplified using the extracted genome DNA obtained above as the template and the universal fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR amplification was performed with an initial denaturation at 98 °C for 5 min followed by 35 cycles of 10 s at 98 °C, 50 s at 50 °C, 1 min at 72 °C, then at 72 °C for 10 min. PCR reaction system was 50 μl: 2 μl each of ITS1, ITS4, and DNA template; 25 μl primeSTAR HS DNA polymerase; and 19 μl ddH_2O.

PCR products were identified by 1% agarose gel electrophoresis, purified using the MiniBEST DNA Fragment Purification Kit (Takara, Japan) according to the manufacturer's instruction, and then cloned into pMD19-T vector by conventional molecular cloning methods. The recombinant vector plasmid was sequenced by Sangon Biotech (Shanghai) Co., Ltd., China.

Full-length sequences of the rRNA-ITS genes which included the full-length ITS and the partial 18S and 28S rRNA sequences were obtained and subjected to sequence alignments in the GenBank Database of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The maximum likelihood (ML) tree was constructed using MEGA 7.0 software, and the bootstrap analyses were performed employing JTT model-based distance matrices generated from 1000 samplings of the alignments.

The pathogenicity of the fungal isolate on silkworms

After the 10 day culture at 25 °C on the PDA solid medium, the conidia of the strain were collected, counted, and suspended in sterile water with 0.1% Tween-80. Using a hemocytometer, the detected concentration of the conidia was 5×10^9 conidia/ml. The conidia suspension was then diluted by 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} with sterile water. Twenty silkworm larvae of the fourth instar were immersed in the conidia suspensions of different concentrations each for 10 s. Meanwhile, a control suspension was prepared with twenty silkworm larvae of fourth instar immersed in sterile water with 0.1% Tween-80. All the silkworms were fed on fresh mulberry leaves in closed boxes with a piece of wet paper in every box to keep it moist during the first 24 h. Then, they were fed on clean mulberry leaves in normal environment and observed every day. We checked the pupae in cocoons to make sure they were not infected during the larval stage, and so to determine whether they were infected during the pupal stage. The dead silkworms were kept in 90% relative humidity and at 25 °C to ensure if the dead silkworms were infected by the isolated strain.

All the aforementioned experiments were repeated at least three times independently. The mortality was statistically analyzed using SPSS 20.0, and the statistical significance was assessed using t-test.

RESULTS

Phylogenetic analysis based on rRNA-ITS gene sequences

PCR product was identified by 1% agarose gel electrophoresis, and the rRNA-ITS of the strain was about 600 bp in size (Fig. 1), which includes the full-length ITS and the partial 18S and 28S rRNA, were sequenced and submitted to GenBank Database of NCBI (Accession number MW167069). The homologous sequences

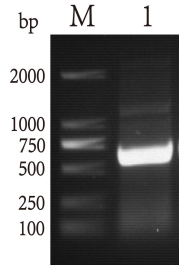

Fig. 1 Gel electrophoresis of rRNA-ITS sequence of the *C. cateniobliqua* Bm1. M: DL2000 DNA Ladder Marker. 1: PCR product of rRNA-ITS of *C. cateniobliqua* Bm1.

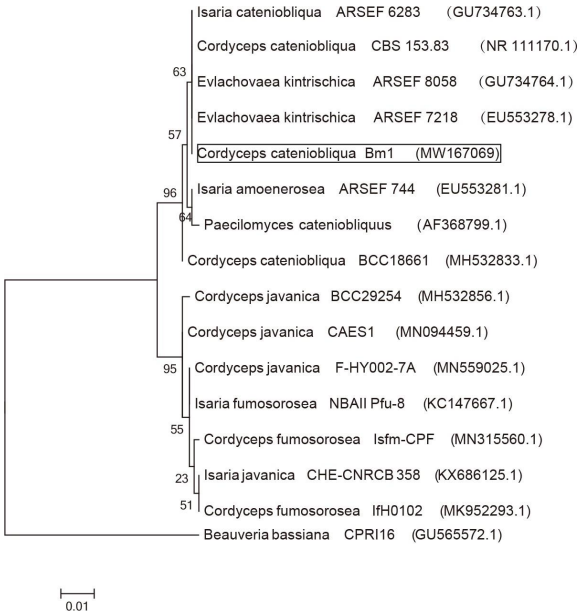

Fig. 2 Phylogenetic tree based on rRNA-ITS sequence of *C. cateniobliqua* Bm1. The tree is constructed using ML method with 1000 bootstrap replicates. Clade credibility values are indicated at the nodes.

from fungal strains were chosen to construct the phylogenetic tree using the Beauveria bassiana as outgroup (Fig. 2). Four fungal strains: *I. cateniobliqua* (ARSEF6283), *Cordyceps cateniobliqua* (CBS153.83), *Evlachovaea kintrischica* (ARSEF8058), and *E. kintrischica* (ARSEF7218), were clustered in the same clade with the Bm1 strain (MW167069). The *E. kintrischica* was found by Borisov and Tarasov as a new species [10]. According to description of *E. kintrischica*, it might be the synonym of *I. cateniobliqua*. Since the *Isaria* genus and the *Evlachovaea* were reclassified as *Cordyceps* in 2017 [6], the Bm1 isolated from the silkworm was probably an isolate of *C. cateniobliqua*.

Morphological evaluations

The Bm1 strain was cultured on the PDA solid medium and Czapek-Dox medium. The colour of the colony on the PDA solid medium was white at first and gradually became pink. The shape of the fungal colony looked like a concentric ring on the PDA medium. The surface of colony was granular (Fig. 3A). The bottom of the colony (back side) was orange red (Fig. 3B) and changed darker after cultured for a long time. The Bm1 strain colony on the Czapek-Dox medium was swell with a granular surface as well. The colour of the colony gradually became pink, and digitate coremia grew out of the middle of the colony (Fig. 3C). The average hyphal growth rate of the Bm1 strain was 3.42 ± 1.24 mm/day with the highest rate at the fourth and the fifth days (Fig. 4). The hyphae were colourless and 0.95–1.62 mm in width and had septa (Fig. 5A). The diameter of colony after cultured for 11 days on PDA medium was 40.39 ± 1.24 mm. The conidiogenous cells were colourless and smooth. The individual cell had flask-like phialide with slightly swollen or columnar base gradually narrow to a distinct neck of (5.06–10.35) μm $\times$ (1.56–2.34) μm in size and verticillate on hypha (Fig. 5B). The Bm1 strain conidia were smooth, colourless, oval, oblong oval or irregularly cylindrical, and (2.93 ± 0.31) μm $\times$ (1.84 ± 0.21) μm in size (Fig. 5C). They were arranged in a broadly 'V'-like manner that resulted in irregularly curved and flat chains (Fig. 5D,E). Although the rRNA-ITS gene sequences of the *C. amoenerosea* and the *C. fumosorosea* were similar to the *C. cateniobliqua*'s, the sizes and shapes of their conidia and the colours of their colonies were different [7]. The characteristic difference between these species was the arrangement mode of conidia. The arrangement of conidia of the *C. cateniobliqua* was irregularly curved and flat chains and in a broadly 'V'-like manner, whereas the arrangement of conidia of the *C. amoenerosea* and the *C. fumosorosea* were linear chains. Consequently, the strain isolated from the silkworm in this study was identified as a strain of *Cordyceps cateniobliqua* and named as *Cordyceps cateniobliqua* Bm1 by combining the result of phylogenetic analysis and the morphological evaluations.

Symptoms of silkworms infected with *Cordyceps cateniobliqua* Bm1

In the early stage of silkworms infected by *C. cateniobliqua* Bm1, the feeding behaviour and the characteristic of cuticle were the same as healthy silkworms. 5 days after infection, several silkworms showed in appetent and molted difficultly. Some black-brown spot appeared on the body surface and mostly around the body segments (Fig. 6A). The infected silkworms spit out the intestinal juice before death. In the early stage of death, the silkworm body was soft, while the colour of the body did not change. Blastospores were

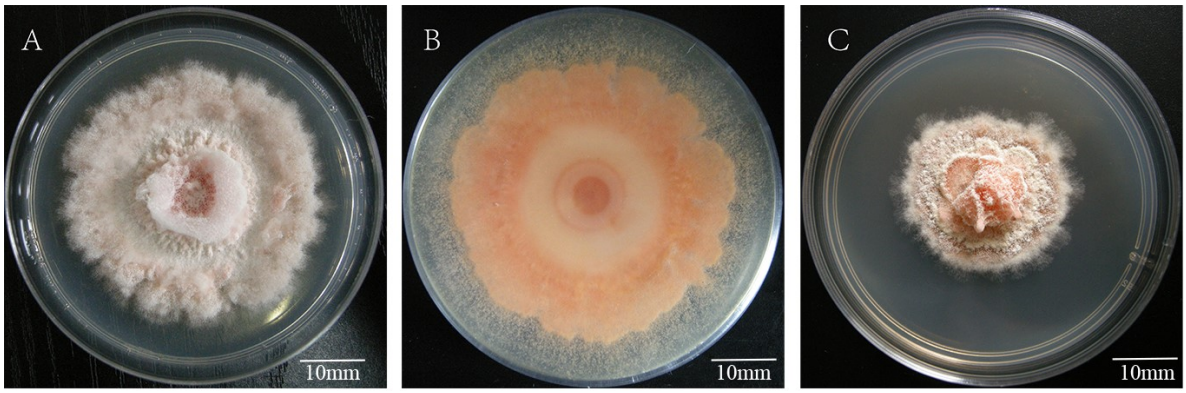

Fig. 3 Colonial morphology of *C. cateniobliqua* Bm1. A: The front of colony of *C. cateniobliqua* Bm1 cultured on PDA solid medium at 25 °C for 10 days. B: The back of colony of *C. cateniobliqua* Bm1 cultured on PDA solid medium at 25 °C for 10 days. C: *C. cateniobliqua* Bm1 was cultured on Czapek-Dox medium at 25 °C for 10 days.

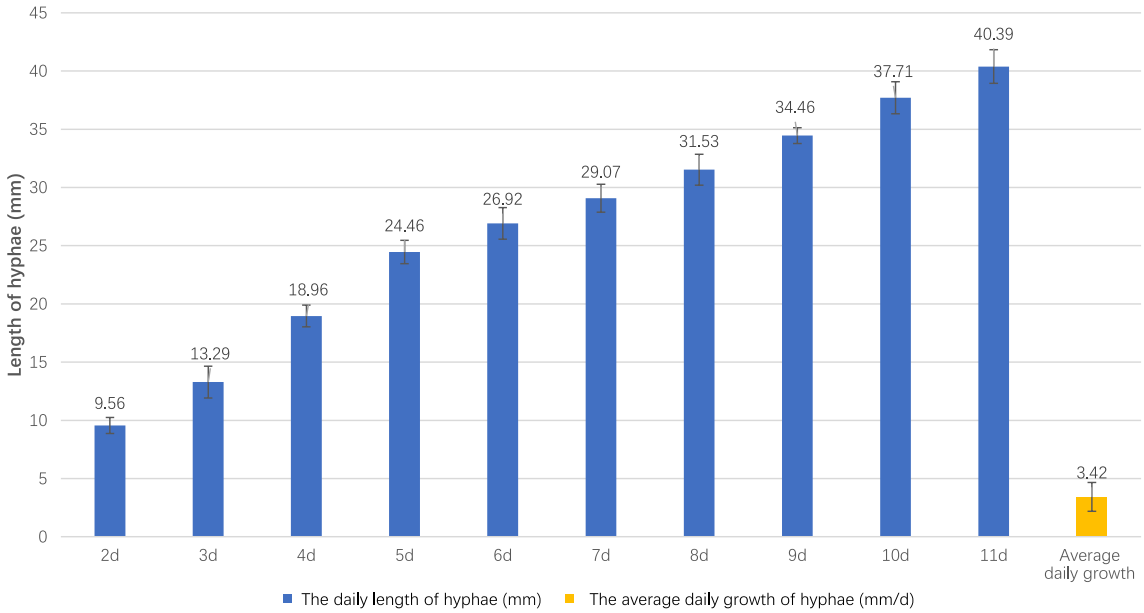

Fig. 4 The hyphal growth of *C. cateniobliqua* Bm1 on PDA medium. The length of hypha is indicated as the mean ± standard deviation of three repeats.

observed in the blood of infected silkworms under the optical microscope (Fig. 5F). At 2 or 3 days after death, the silkworm body was rigid, and the cadaveric colour was reddish (Fig. 6B). At 5 to 6 days after death, white conidia appeared on the cadaveric surface (Fig. 6C), and white aerial mycelia subsequently grew out of the silkworm somite (Fig. 6D). In the humid environment, the aerial mycelia grew longer and were digitate; and they gradually became reddish. At 8 to 10 days after death, the cadaveric surfaces were fully covered with white conidia (Fig. 6E). The course of disease was long; some silkworms had no symptoms during the larval phase, but they died during the pupal stage. The dead pupae would become rigid, and the symptoms of the infected pupae were the same as the infected silkworms (Fig. 6F).

The pathogenicity of *Cordyceps cateniobliqua* Bm1 on silkworms

The silkworm larvae were immersed in the conidia suspension of five different concentrations. The mortality rates of the silkworm infected by *C. cateniobliqua* Bm1 increased with the increases of concentration of conidial suspension (Fig. 7). None of the silkworms got infected after being immersed in the conidia suspensions of 5×10^5 and 5×10^6 conidia/ml; whereas the silkworms immersed in the higher concentrations of conidia suspension began to die on the fifth day, and the mortality rates increased with the increases of concentration of conidial suspension. On the tenth

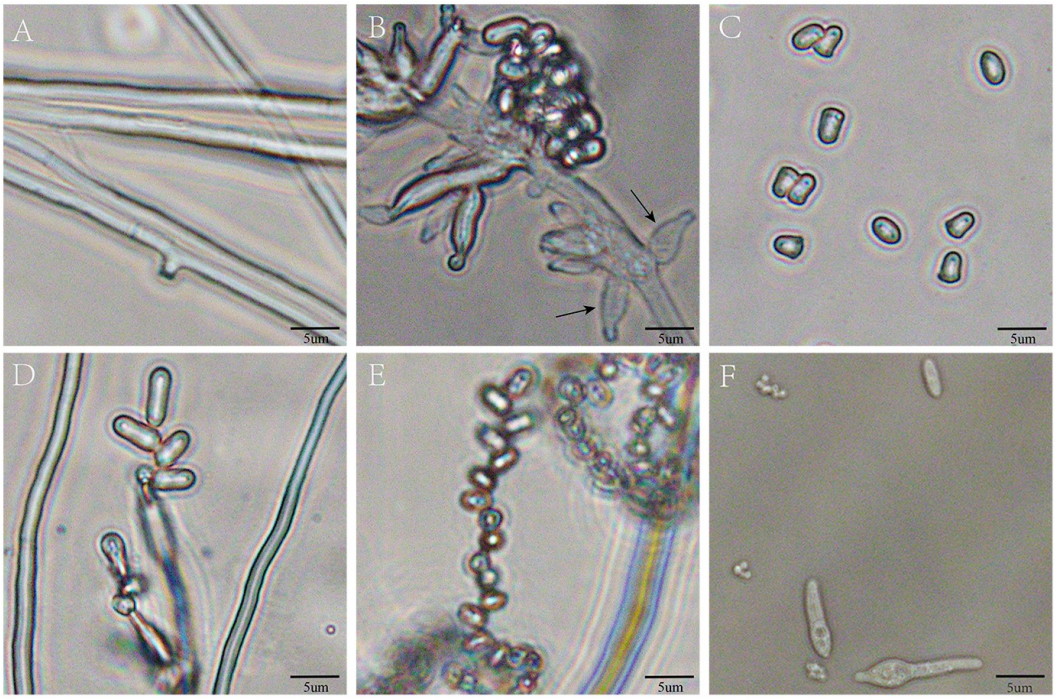

Fig. 5 Optical microscope (OM) images of *C. cateniobliqua* Bm1. A: Hyphae of *C. cateniobliqua* Bm1. B: The structure that produces conidia. Black arrow: the conidiogenous cells. C: Conidia of *C. cateniobliqua* Bm1. D-E: The arrangement of conidia. F: The blastospores in the blood of infected silkworms.

Fig. 6 Symptoms of silkworms infected by fungal strain *C. cateniobliqua* Bm1 at different stages. A: The early stage of silkworm infected by *C. cateniobliqua* Bm1, some black-brown spot appeared on the cuticle. B: At 2–3 days after dead, the cadaveric color became reddish. C: At 5–6 days after dead, white aerial mycelia grew out of the silkworm somite. D: At 8–10 days after dead, the aerial mycelia grew longer, and some white conidia appeared. E: In the humid environment, the aerial mycelia grew longer and gradually became reddish. F: The symptom of infected pupa.

Table 1 Pathogenicity of *C. cateniobliqua* Bm1 on the silkworm.

Strain	Concentration of fungi (10^9 conidia/ml)	Corrected Mortality (%)	Regression equation	LC_{50} (10^9 conidia/ml)	95% confidence interval
C. cateniobliqua Bm1	0.005 0.05 0.5 5	0 25 30 70	$y = 0.0934\ln(x) + 0.4847$ $R^2 = 0.9183$	1.1780	0.5365–2.5864

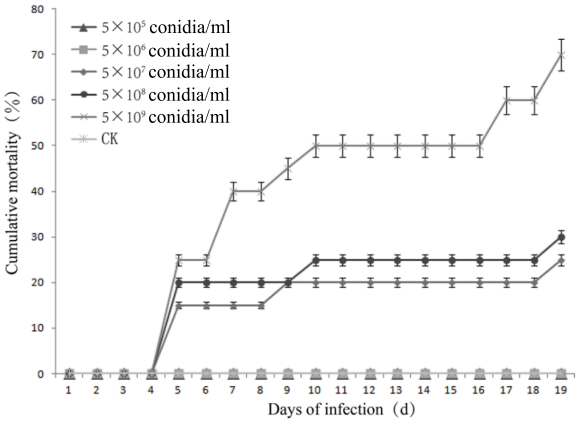

Fig. 7 The mortality of *C. cateniobliqua* Bm1 to the silkworm. 1–12 days, the larval phase; 12–19 days, the pupal stage.

day after infection, the mortality rates of the silkworms were the highest during the larval phase. The mortality rates of the silkworms treated with 5×10^7, 5×10^8, and 5×10^9 conidia/ml were 20%, 25%, and 50%, respectively during the larval stage. On the seventeenth day after infection, we checked the silkworm cocoons to confirm whether any silkworm got sick during the pupal stage; and on the nineteenth day, the mortality rates of the silkworms treated with the three concentrations reached ultimately to 25%, 30%, and 70%, respectively (Fig. 7). The mortality rates were analyzed by SPSS 20.0 with the method of logarithmic in curve estimation to calculate the regression equation, which was $y = 0.0934\ln(x) + 0.4847$. The LC_{50} was 1.1780×10^9 conidia/ml ($p < 0.05$) as showed in Table 1.

DISCUSSION

In this study, a fungal pathogen was isolated from infected silkworms. The ITS region was used as a universal DNA barcode marker for Fungi [11]. The morphological and the ITS sequence analysis technique were used to identify the newly isolated fungal strain. We found that the newly isolated Bm1 was clustered in the same clade with *C. cateniobliqua* and *E. kintrischica* based on rRNA-ITS gene sequence, and that the morphological characteristics of the Bm1 are similar to the *C. cateniobliqua*'s the most. By the results of the phylogenetic analyses and the morphological evaluations, the strain isolated from silkworms was identified as *C. cateniobliqua*.

Entomogenous fungi such as *Beauveria*, *Metarhizium* and *Verticilliumlecantii* can be used as biological insecticide [12], while *Cordyceps militaris*, *Ophiocordyceps sinensis*, and *C. cicadae* can be used as medicinal fungi [13]. As a kind of entomogenous fungi, the *Cordyceps* genus not only has been used in biological control, but also plays an important role in the fields of medicine and functional food. The *C. farinose* has extensive pharmacological action, such as cough expectorant and anticancer [7, 14]. *C. tenuipes* can enhance immunity and has antibacterial activity [15, 16]. *C. fumosorosea* can be used as a kind of biological agents; i.e., the highly pathogenic *C. fumosorosea* strain can infect whitefly and aphid and has already been used for greenhouse pest control [17–19]. The white muscardine is a common fungal silkworm disease. When silkworms were infected by *Beauveria bassiana*, the cadavers of dead larvae would be mummified. These mummified cadavers could be processed into a traditional Chinese medicine; for example, *Bombyx batryticatus* has been used for the treatment of epilepsy, headaches, cough, tonsillitis, convulsions, thyroid adenoma, and asthma [20–22]. A research showed that the *C. cateniobliqua* can infect *Ephestia elutella* [23], and the fermentation broth of *C. cateniobliqua* can inhibit *Candida albicans*. Our study was the first to isolate *C. cateniobliqua* strain from silkworms. The LC_{50} of the white muscardine pathogen was about 10^5 conidia/ml, and the LT_{50} was 4–5 days [24]. Although the pathogenicity of *Cordyceps cateniobliqua* Bm1 to the silkworm was low and the disease course was long, the complex of entomogenous fungi and silkworms probably has some medicinal substance. So, it is worthwhile to study if the *C. cateniobliqua* Bm1 has potential medicinal value.

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