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Identification of entomopathogenic fungus Cordyceps cicadae isolated from soil using common cutworm Spodoptera litura (Lepidoptera: Noctuidae) as bait and its high virulence comparable to generalist Metarhizium anisopliae complex

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1 Original article for Fungal Biology

2 Title

3 Identification of entomopathogenic fungus *Cordyceps cicadae* isolated from soil using common cutworm

4 *Spodoptera litura* (Lepidoptera: Noctuidae) as bait and its high virulence comparable to generalist *Metarhizium*

5 *anisopliae* complex

6

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13

14 **Abstract**

15 The common cutworm (*Spodoptera litura,* Lepidoptera: Noctuidae) is one of the most widespread pest insects

16 affecting various crops in Asian countries. To screen promising biological control agents for this pest, we isolated 49

17 potential entomopathogenic fungal isolates from 25 soil samples using *S. litura* and four additional insect species as

18 baits. The results revealed a high occurrence (24%) of *Cordyceps cicadae* in soil through the bait method with all five

19 insect baits, following the *Metarhizium anisopliae* complex (52%), despite *C. cicadae* generally being known as a

20 specialist entomopathogenic fungus of cicada nymphs. Molecular phylogenetic analysis demonstrated that *C. cicadae*

isolates from soil and natural cicada nymphs clustered together at a terminal node with previously reported *C. cicadae* from cicada nymphs and *C. lepidopterorum* from lepidopteran larvae. Virulence assays against last-instar larvae of *S. litura* revealed significant variability in virulence among *C. cicadae* strains derived from soil and cicada nymphs. Among these, *C. cicadae* S17, isolated from soil using *S. litura* as bait, exhibited virulence comparable to the most virulent strains of the *M. anisopliae* complex and was also virulent against third-instar larvae and pupae. Our findings indicate that *C. cicadae* exhibit a broader host range than previously recognized, with potential applications in biological control for larvae and pupae of *S. litura*.

Keywords: Cordycipitaceae, Cicadae, Phylogenetic analysis, *Cordyceps lepidopterorum*, Biological control

31 **Highlight**

32 - *Cordyceps cicadae* is recognized as a fungal parasite specific to cicada nymphs

33 - Unexpectedly, *C. cicadae* was isolated from soil with 5 baits including cutworm

34 - Molecular phylogenetic analysis confirmed the species identification

35 - The fungus exhibited high virulence against young larvae and pupae of the cutworm

36 - Its virulence was comparable to generalist *Metarhizium anisopliae* complex

37

Graphical Abstract (for review)

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Cordyceps cicadae

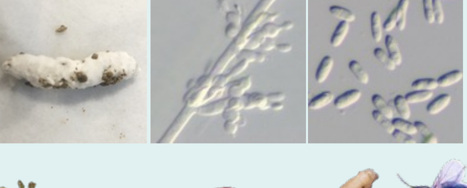
Traditionally recognized as **fungal parasite of cicada nymph**

Broad host range

C. cicadae in natural soil **infected 5 different insects including cutworm**

Spodoptera litura

- Common cutworm
- a serious crop pest



High biocontrol potential for lepidopteran pest

Both *C. cicadae* from soil and cicada nymph exhibited **high virulence against cutworm, as comparable to *Metarhizium anisopliae* complex**, a generalist entomopathogen

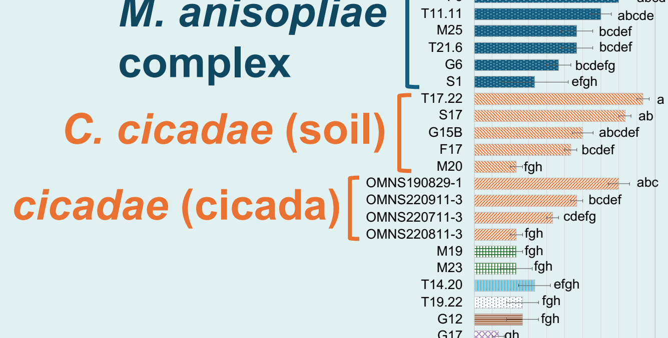


Figure 1

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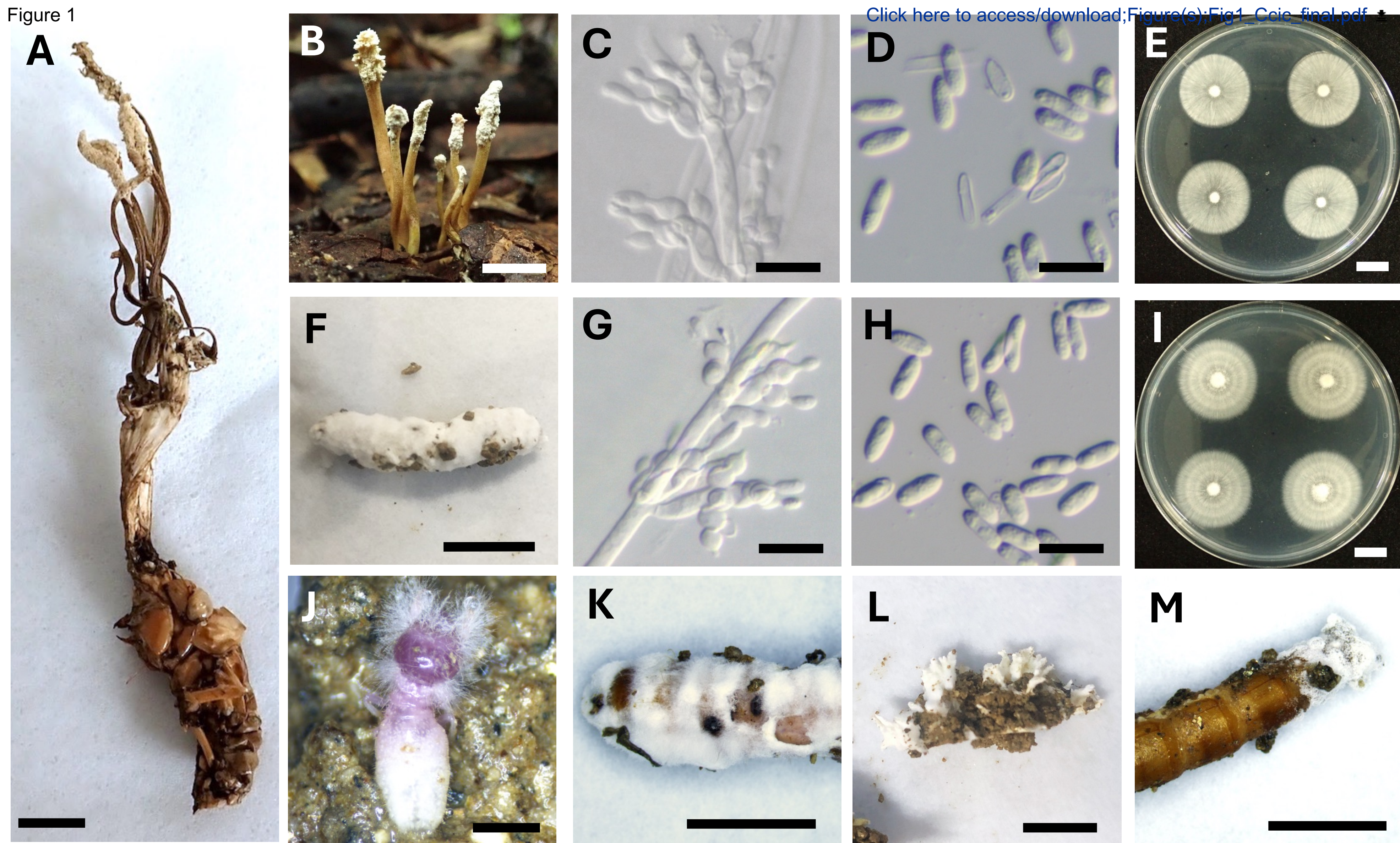

Figure 2

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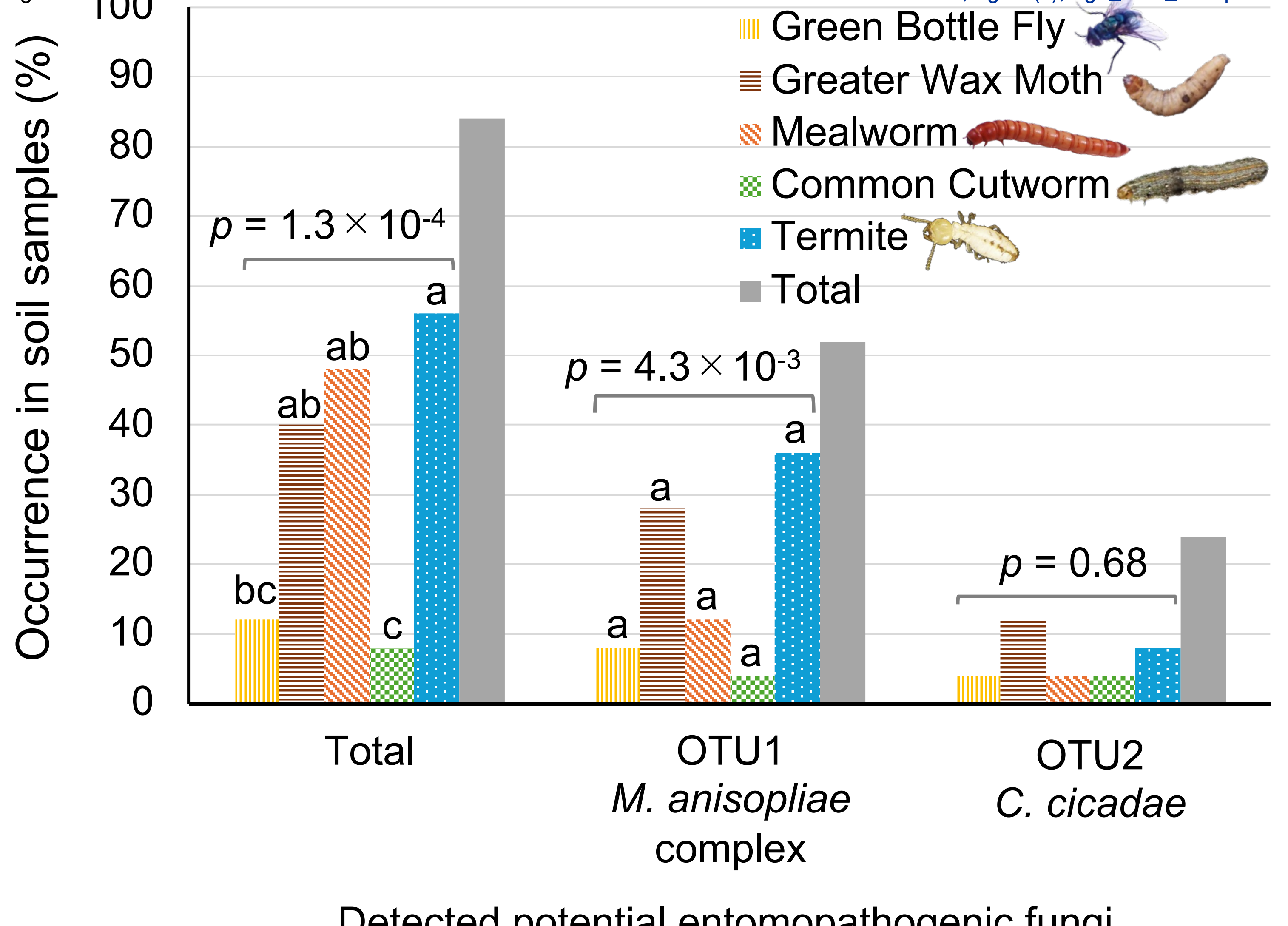

Figure 3

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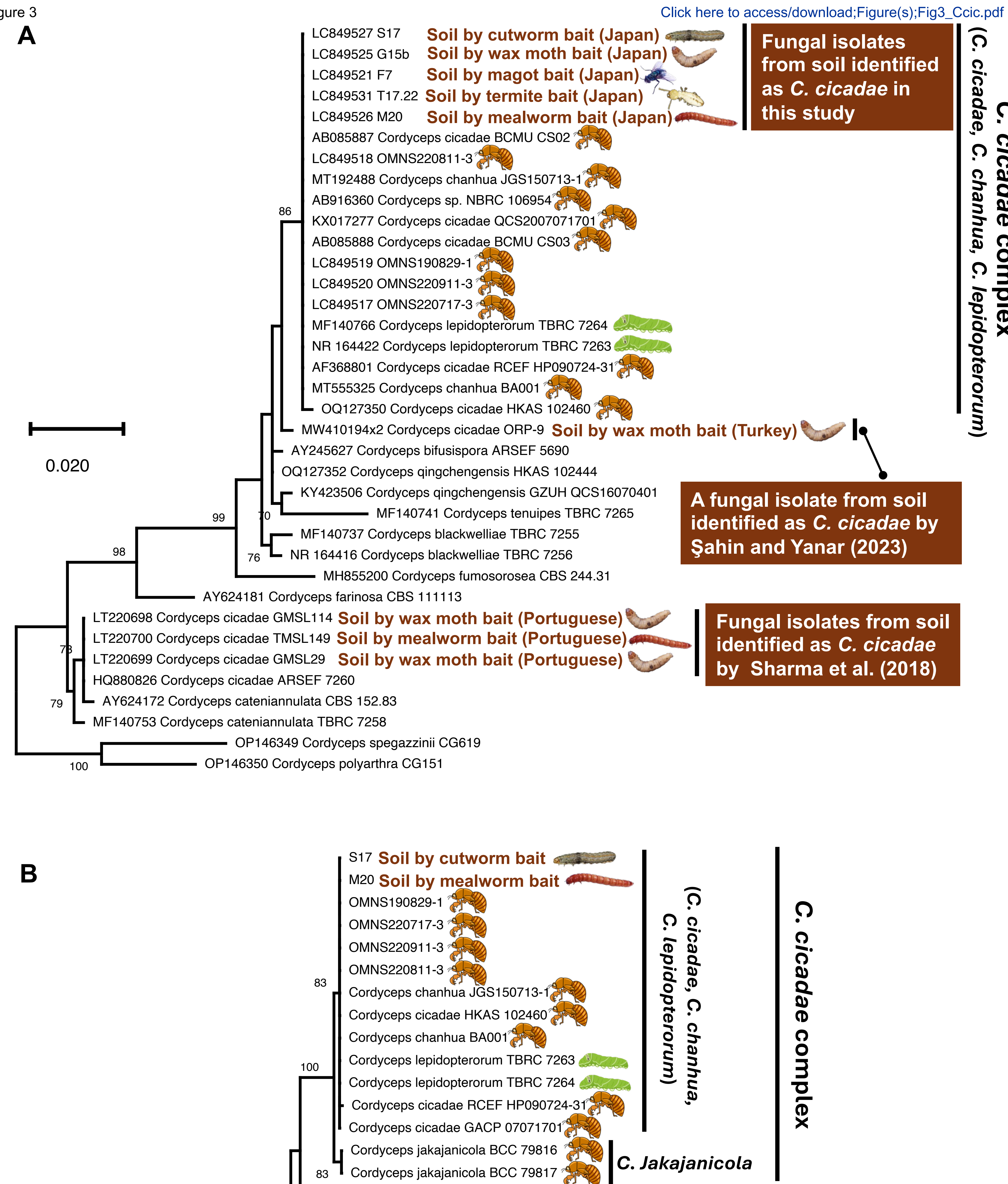

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Fig. 4

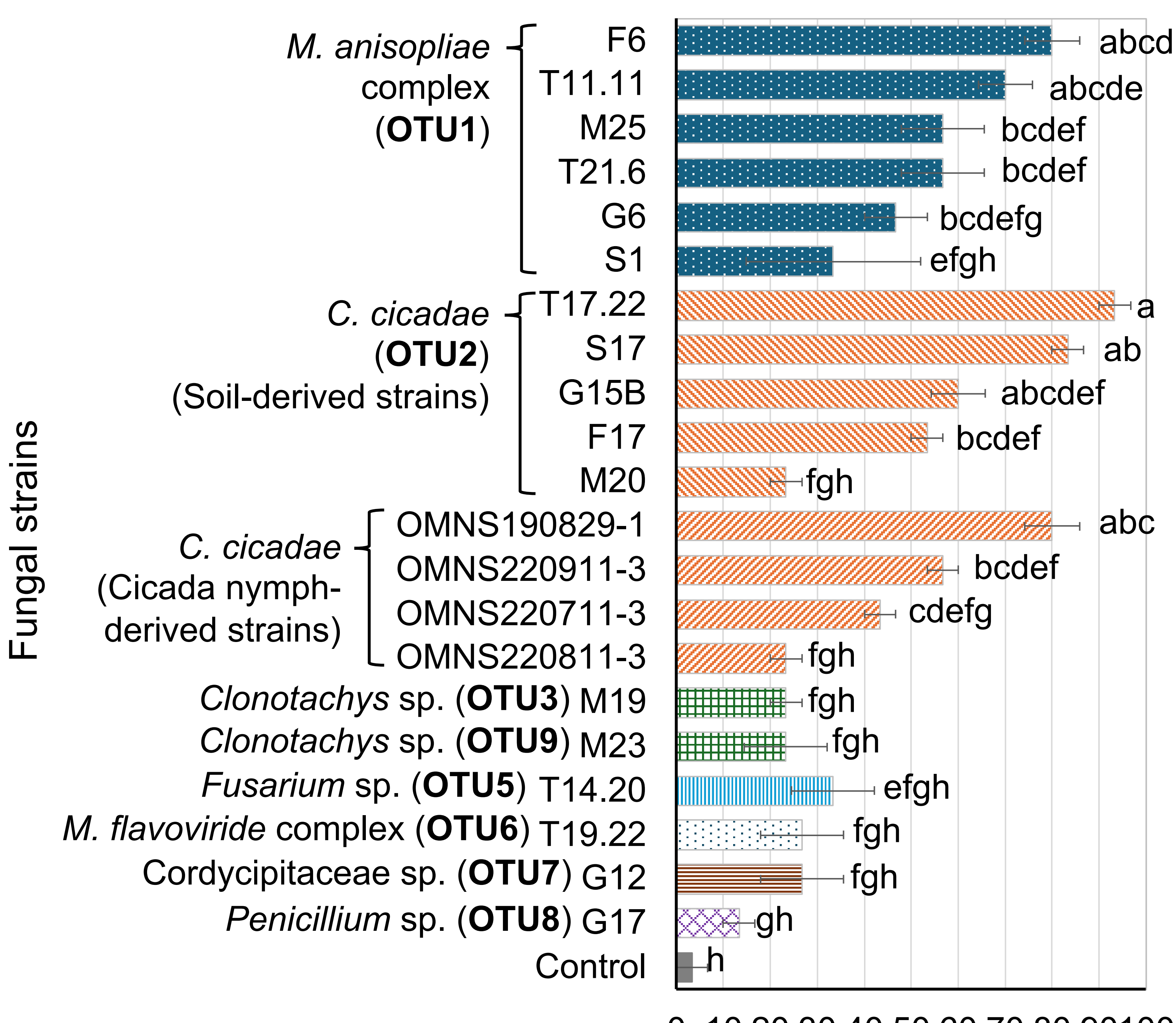

Figure 5

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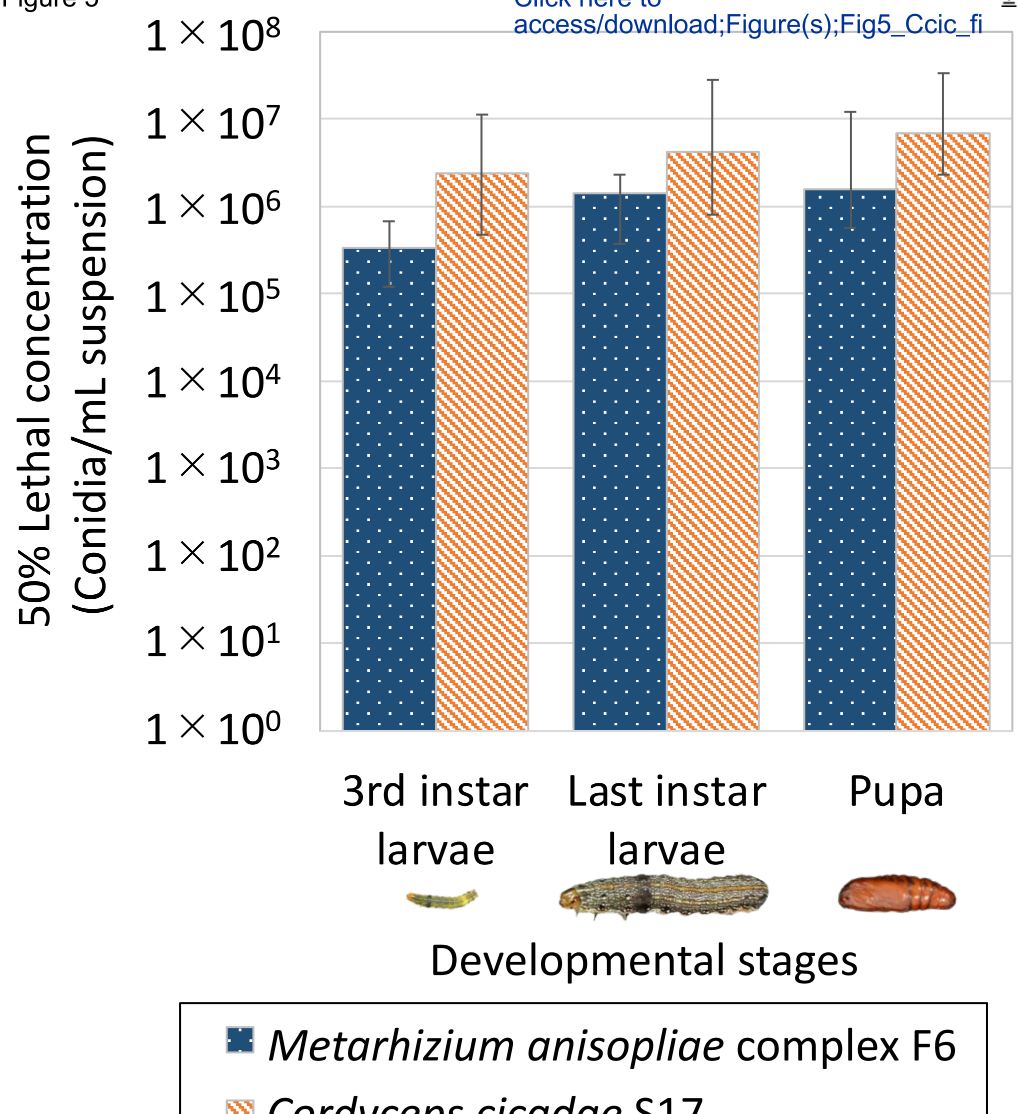

Table(s)

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Table 1. Fungal strains used for virulence assay.

Species (OTU)	Strain	Isolation source[1]	Location	Genbank Accession No. rDNA ITS
Metarhizium anispopliae complex (OTU1)	F6	Soil (Maggot)	KUI[2], Fukuoka, JP	LC849542
	T11.11	Soil (Termite)	KUI, Fukuoka, JP	LC849554
	M25	Soil (Mealworm)	KUI, Fukuoka, JP	LC849555
	T21.6	Soil (Termite)	KUI, Fukuoka, JP	LC849561
	G6	Soil (Wax moth)	KUI, Fukuoka, JP	LC849543
	S1	Soil (Cutworm)	KUI, Fukuoka, JP	LC849559
Cordyceps cicadae (OTU2)	T17.22	Soil (Termite)	KUI, Fukuoka, JP	LC849531
	S17	Soil (Cutworm)	KUI, Fukuoka, JP	LC849527
	G15b	Soil (Wax moth)	KUI, Fukuoka, JP	LC849525
	F7	Soil (Maggot)	KUI, Fukuoka, JP	LC849521
	M20	Soil (Mealworm)	KUI, Fukuoka, JP	LC849526
	OMNS190829-1	Cicada nymph (*Meimuna opalifera*)	KUI, Fukuoka, JP	LC849519
	OMNS220717-3	Cicada nymph	Oita, JP	LC849517
	OMNS220811-3	Cicada nymph	Kagoshima, Japan	LC849518
	OMNS220911-3	Cicada nymph	Fukuoka, Japan	LC849520
Clonostachys sp. (OTU3)	M19	Soil (Mealworm)	KUI, Fukuoka, JP	LC849567
Fusarium sp. (OTU5)	T14.20	Soil (Termite)	KUI, Fukuoka, JP	LC849537
M. flavoviride complex (OTU6)	T19.22	Soil (Termite)	KUI, Fukuoka, JP	LC849564
Cordycipitaceae sp. (OTU7)	G12	Soil (Wax moth)	KUI, Fukuoka, JP	LC849539
Penicillium sp. (OTU8)	G17	Soil (Wax moth)	KUI, Fukuoka, JP	LC849540
Clonostachys sp. (OTU9)	M23	Soil (Mealworm)	KUI, Fukuoka, JP	LC849532

[1] For soil isolates, bait insects are presented in the parenthesis

[2] ITO campus, Kyushu University

38 **Introduction**

39 *Spodoptera litura* (Lepidoptera: Noctuidae), generally known as the common cutworm, is a major insect pest
40 affecting crops in the Asian tropics (Lin et al. 2019; Tojo et al. 2013). For instance, in Vietnam, *S. litura* is distributed
41 across all regions and is one of the seven primary pests that damage tomato plants in northern Vietnam (Nguyen et al.
42 2006). Economic losses caused by this pest range from 25.8 to 100% in certain cases (Sahu et al. 2020). Among its
43 life stages—eggs, larvae, pupae, and adults—only the larvae cause significant damage, primarily during the later instar
44 stages. At high population densities, larvae can completely defoliate leaves through excessive chewing (Vijaya et al.
45 2016). Moreover, in sandy soils, larvae hiding during the day can potentially damage groundnut pods (EFSA Panel
46 on Plant Health, 2019). Due to its polyphagous nature, which includes feeding on at least 389 species of industrial
47 crops (Brown and Dewhurst, 1975), *S. litura* is capable of invading new areas and quickly adapting to diverse natural
48 and climatic conditions.

49 In recent years, farmers have relied heavily on chemical pesticides as the primary method for controlling *S.*
50 *litura* (Srivastava et al., 2018). However, the rapid development of resistance to many commonly used insecticides,
51 particularly pyrethroids and carbamates, has rendered chemical control increasingly ineffective (Kranthi et al., 2002).
52 Additionally, the use of chemical pesticides poses significant risks to the environment, human health, and animal
53 welfare (Beketov et al., 2013). Consequently, biological control (biocontrol) has emerged as a crucial alternative to
54 chemical pesticides, offering a means to mitigate these adverse effects. Among biocontrol methods, entomopathogenic
55 fungi (EPF), such as *Beauveria* spp., *Metarhizium* spp., and *Cordyceps* spp. (partially formerly known as
56 *Paecilomyces* and *Isaria*), are particularly promising, as they can infect all life stages of their insect hosts (Hajek and
57 St. Leger, 1994).

58 The soil environment serves as an important reservoir for EPFs (Keller et al. 1989). For isolation of EPFs
59 from soil, traditionally two primary methods have been used to isolate EPFs from soil, i.e., the plating method with
60 semi-selective medium and the insect bait method using wax moth larvae (*Galleria mellonella*, Lepidoptera:
61 Pyralidae) or mealworms (*Tenebrio molitor*, Coleoptera: Tenebrionidae) (e.g., Nishi et al. 2017a; Steinwender et al.
62 2014; Zimmermann, 1986). Theoretically, the most efficient method for screening entomopathogenic fungi pathogenic
63 to *S. litura* from soil is the bait method using *S. litura* itself, as this approach uses pathogenicity to the target insect as
64 a selection criterion. However, to our knowledge, neither *S. litura* nor its close relatives have been used for isolating
65 EPFs from soil.

66 In a screening for entomopathogenic fungi from soil using the bait method with *S. litura* and four additional
67 insect species, we found that *C. cicadae* predominantly occurred and was isolated from soil using *S. litura* as bait.
68 This finding was unexpected, as *C. cicadae* is generally regarded as a specialist parasitic fungus of cicada nymphs
69 (e.g., Aoki, 2003; Isono, 2021). These results suggest the potential of *C. cicadae* as a biological control agent against
70 *S. litura*, although its virulence against *S. litura* is not well understood. With respect to the current taxonomic status
71 of *C. cicadae*, closely related species have been recently proposed through multi-locus DNA sequence analysis.
72 Therefore, multi-locus phylogenetic analysis is necessary for the accurate identification of *C. cicadae*.

73 In this study, we focused on *C. cicadae* isolated from soil using *S. litura* as bait. We report the occurrence of
74 *C. cicadae* from soil via insect bait methods, the phylogenetic positions of soil-derived *C. cicadae* strains, and the
75 virulence of these strains against *S. litura* in comparison with other EPF species. For further identification and
76 virulence comparisons, *C. cicadae* derived from natural cicada nymphs were also examined to elucidate the

77 phylogenetic and virulence differences between *C. cicadae* isolated from soil and *C. cicadae* isolated from cicada

78 nymphs.

79

80 **Materials and Methods**

81 **Rearing and preservation of *S. litura***

82 *Spodoptera litura* (the Ishihara strain), kindly provided by Ishihara Sangyo Kaisha, Ltd. (Osaka, Japan), was

83 used for the virulence assay. Larvae were reared in the laboratory at 25 ± 3°C, as per the method proposed by

84 Gebreslasie et al. (2023). First- to third-instar larvae were maintained on an artificial diet (250 g soybean, 250 g wheat

85 bran, 100 g dried yeast, 32.5 g agar, 10 g ascorbic acid, 3.5 g sorbic acid, 7.5 g propionic acid, and 1000 g RO water;

86 for a total of 2400 g of diet). From fourth instar onward, artificial diet was replaced with fresh cabbage leaves, which

87 were replenished every 48 h. Pupation occurred in vermiculite, and the pupae were transferred to plastic containers

88 maintained at 25 ± 3°C with a 14L:10D photoperiod. Emerging adults were fed a 10% honey solution. To propagate

89 the next generation, egg masses were placed on regular copy paper, transported to separate plastic containers, and fed

90 an artificial diet.

91

92 **Soil sample**

93 Twenty-five soil samples (approximately 5–10 cm in depth) were collected after removing surface litter from

94 two forested areas on Ito Campus, Kyushu University (Fukuoka, Japan). Each soil sample was placed in a plastic bag

95 and stored at 4°C. Prior to use, all soil samples were sieved through a 4-mm mesh to remove impurities.

96

97 **Isolation of EPFs from the soil using the insect bait method**

98 Potential EPFs were isolated from the collected soil samples using five insect species as baits; the common
99 cutworm (*S. litura,* last-instar larvae), greater wax moth (*Galleria mellonella*, larvae 1.0–1.5 cm in length, obtained
100 from a pet solution store), mealworm (*Tenebrio molitor*, larvae 2.0–2.5 cm in length, obtained from a pet solution
101 store), subterranean termite (*Reticulitermes speratus,* workers collected on the Kyushu University campus, Fukuoka,
102 Japan), and green bottle fly (*Lucilia sericata,* last-instar larvae, obtained from a fishing solution store). The isolation
103 procedure was as per the method described by Nishi et al. (2011, 2017b) for subterranean termites and Meyling (2007)
104 for the other insects. Bait insects were released onto each soil sample (24 workers for subterranean termites and 8
105 larvae for other insects per soil sample). Insect cadavers were inspected daily for the presence of aerial mycelium.
106 Potential fungal pathogens observed on the cadavers were microscopically examined and transferred onto potato
107 dextrose agar medium (PDA, Nissui, Tokyo, Japan). Colony morphology and conidiogenesis were re-examined to
108 confirm that the fungi corresponded to those colonizing the insects.

109

110 **Cicada-nymph-derived *C. cicadae***

111 Four *C. cicadae* strains derived from cicada nymphs were used in this experiment: OMNS190829-1,
112 OMNS220717-3, OMNS220811-3, and OMNS220911-3 (Table 1). These EPFs were isolated from conidial masses
113 at the tips of synemata produced on subterranean cicada nymphs (Hemiptera: Cicadidae) in Japan (Fig. 1A–E).

114

115 **DNA sequencing, identification, and phylogenetic operational taxonomic unit (OTU) grouping**

116 Crude genomic DNA from the fungal isolates was prepared by suspending fresh mycelium from PDA
117 cultures in TE buffer. For the identification of soil isolates, the DNA sequence of the ITS1-5.8S rDNA-ITS2 (ITS)
118 region was determined. The PCR mix consisted of the following: 10% (v/v) crude DNA; 1× KOD One® PCR Master
119 Mix -Blue (TOYOBO, Osaka, Japan); and 3 pmol of each primer, in a total of 10 µL. The primer set ITS5 and ITS4
120 (White et al. 1995) was used for the reaction. The PCR program and primer details are summarized in Supplementary
121 Table 1. The PCR products were purified using MagExtractor™–PCR & Gel Clean up– (TOYOBO, Osaka, Japan).
122 Sanger DNA sequencing of the PCR products was performed by GENEWIZ from Azenta Co. (Massachusetts, United
123 States). Single-strand sequencing was performed using the reverse primer (ITS4). Species- or genus-level affiliation
124 of the fungal isolates was inferred via BLASTn of the ITS sequences using NCBI BLAST
125 (https://blast.ncbi.nlm.nih.gov/). For the OTU grouping of the isolates, multiple sequence alignments of the ITS
126 sequences were conducted using MUSCLE in MEGA X (Tamura et al., 2021). UPGMA clustering was then performed
127 on the aligned sequences using MEGA X, with all ambiguous positions removed for each sequence pair (pairwise
128 deletion option). All other settings were left as default. OTU grouping was performed with p-distance=0.03 as the
129 threshold (Garnica et al., 2016).
130

131 **Phylogenetic analysis of *C. cicadae* with reference strains**

132 To determine the phylogenetic positions of *C. cicadae* isolates from soil and cicada nymphs, phylogenetic
133 analyses were conducted using rDNA ITS and a combined dataset of three loci (LSU, RPB2, and TEF). DNA
134 sequences of the ITS region were determined for all nine *C. cicadae* isolates (four cicada-derived isolates and five
135 soil-derived isolates), while sequences of the three additional loci were determined for six isolates, four from cicada

136 two from soil origins. PCR and DNA sequencing were performed as described above, using the conditions outlined

137 in Supplementary Table S2.

138 The following primers were used for gene amplification: ITS5 and ITS4 for ITS (White et al. 1990), LROR

139 and LR5 for LSU (Vilgalys and Hester, 1990), 983F and 2218R for TEF (Rehner and Buckley, 2005), and RPB2-

140 5F2 and RPB2-7Cr for RPB2 (Castlebury et al. 2004). DNA sequencing of *C. cicadae* isolates was performed for

141 both strands.

142 The dataset for the phylogenetic analysis of the ITS region consisted of 36 sequences, including 9 newly

143 obtained sequences and 27 reference sequences. The dataset for the three loci (LSU, RPB2, TEF) comprised 27

144 concatenated sequences, including 18 newly obtained sequences. Reference sequence data were obtained from 18

145 studies, as detailed in Supplementary Table 3 (Crous et al. 2019; Dong et al. 2022; Grudniewska et al. 2014; Kepler

146 et al. 2012; Kepler et al. 2017; Li et al. 2008; Li et al. 2021; Luangsa-ard et al. 2005; Mongkolsamrit et al. 2018;

147 Rehner et al. 2011; Şahin and Yanar 2021; Sung et al. 2007; Sharma et al. 2018; Tasanathai et al. 2016; Wang et al.

148 2020; Wei et al. 2022; Yokoyama et al. 2004; Zha et al. 2019). *Cordyceps polyarthra* CG151 and *C. spegazzinii*

149 CG619 were designated as outgroup taxa for ITS analysis, while *C. cateniannulata* CBS 152.83 served as the outgroup

150 for the combined dataset.

151 Multiple sequence alignments were performed using MUSCLE (Edgar, 2004), implemented in MEGA X

152 (Stecher *et al*. 2020) with default settings. The alignment lengths were 507 bp for ITS, 743 bp for LSU, 749 bp for

153 RPB2, and 936 bp for TEF. Because DNA sequence information for type materials of *C. cicadae* is unavailable, RCEF

154 HP090724-31 and HKAS 102460 (Kepler et al. 2017, Wei et al. 2022) were designated as reference strains for *C.*

155 *cicadae* identification. These strains were selected based on the availability of DNA sequence information for multiple

156 loci and the availability of morphological data for HKAS 102460 described in Wei et al. (2022). Maximum likelihood
157 phylogenetic analyses were conducted using the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at, Trifinopoulos
158 et al. 2016). For the ITS dataset, the ITS1, 5.8S rDNA, and ITS2 regions were analyzed as separate partitions. Similarly,
159 TEF and RPB2 loci were partitioned based on codon positions (codon1, codon2, and codon3). The best-fitting
160 substitution models for each partition were selected based on the Bayesian information criterion (BIC) using IQ-TREE
161 with free rate heterogeneity; ITS1 (K3Pu+F+G4), 5.8S rDNA (TNe), ITS2 (TPM2u+F+I), LSU (HKY+F+I), RPB2
162 codon1 (TN+F), RPB2 codon2 (F81+F), RPB2 codon3 (K2P), TEF codon1 and codon2 (F81+F+I), and TEF codon3
163 (TPM3u+F+I). Branch supports were evaluated using 1000 ultrafast bootstrap (Minh et al. 2013). All other settings
164 were left as default. The resulting Newick-format tree files were edited and visualized using MEGA X.
165
166 Conidial measurement
167 For morphological identification of *C. cicadae*, conidial dimensions were measured. Conidia of *C. cicadae*
168 strains were collected from 14–21-day-old culture on PDA and mounted on a microscope slide with a drop of sterile
169 distilled water containing 0.05% Tween 80. The conidia were observed under a microscope Nikon OPTIPHOT-2
170 (Nikon, Tokyo, Japan) with a differential interference contrast attachment and photographed with a microscope digital
171 camera WRAYCAM-EL510 (Wraymer Inc., Osaka, Japan). The length and width of 20 randomly selected conidia on
172 the images were measured with ImageJ 1.53a (National Institute of Health, USA).
173
174 **Virulence assay**

175 The virulence of representative fungal strains from each OTU against *S. litura* was assessed as per the method
176 proposed by Gebreslasie et al. (2023). The assay was conducted in two stages. First, the virulence against last-instar
177 larvae was evaluated based on mortality after inoculation with a single concentration of the inoculum suspension
178 (1×10^8 conidia/mL). Second, virulence against third-instar larvae, last-instar larvae, and pupae was assessed by
179 calculating the LC_{50} values for selected fungal strains from the first test. Conidia were harvested from 14–15-day-old
180 fungal cultures grown on agar and suspended in sterile RO water containing 0.05% Tween 80. The suspension was
181 vigorously mixed using a vortex mixer and filtered through sterile cotton to remove hyphal fragments. The conidial
182 concentration was determined using a hemocytometer and adjusted to 1×10^8 conidia/mL or serially diluted by a series
183 of 10 (1×10^4 to 1×10^8 conidia/mL). Larvae and pupae (within three days after molting or pupation) were inoculated
184 by dipping them into 10 mL of the conidial suspension in a 50 mL centrifuge tube for 10 s. The inoculated individuals
185 were then dried on a paper towel and transferred to plastic containers (five larvae or ten pupae per container) lined
186 with a moistened paper towel. A 10 mL suspension of 0.05% Tween 80 served as the control (mock inoculation). The
187 containers were maintained at 25 ± 1°C under a 14L:10D photoperiod. The inoculated larvae were fed fresh cabbage
188 leaves and transferred to new containers every 48 h. Individuals were inspected every other day for up to 10 days post-
189 inoculation, and cadavers were transferred to separate containers as they occurred. The number of cadavers was
190 recorded every other day. Each replicate consisted of ten individuals, and the experiment was repeated three times
191 using freshly prepared inoculum. LC_{50} values were calculated based on mortality on the 10th day post-inoculation.
192

193 **Statistical analysis**

194 The occurrence of potential EPFs from soil was compared among the different bait insects using Cochran's
195 Q test by function "cochrans.q" from library "nonpar" in R (R Core Team, 2022). Pairwise comparisons were
196 conducted using McNemar test by function "mcnemar.test" from library "stats" in R, with *p*-value adjustment
197 according to the Benjamini–Hochberg method by function" p.adjust" from library "stats" in R. In the virulence assay,
198 mortality data were collected up to 14 days post-treatment. The mean arcsine-transformed 14-day mortality rates from
199 three replicates were compared among fungal strains using Tukey's HSD test at a 5% significance level by function
200 "glht" from library "multcomp" in R.

201

202 **Results**

203 **Isolation and identification of EPFs from the soil**

204 Forty-nine potential EPFs were isolated from 25 soil samples (Supplementary Table 1). The naming
205 convention for fungal strains was based on the type of bait insect used; the first letter represents the bait insect (F:
206 green bottle fly; G: greater wax moth; M: mealworm; T: termite; S: *S. litura*), and the number indicates the soil sample
207 number. These EPFs were grouped into nine OTUs based on the UPGMA analysis of the ITS sequence data with p-
208 distance=0.03 as the threshold (Supplementary Fig. 1). The nine OTUs were identified as follows: *M. anisopliae*
209 complex (OTU1), *C. cicadae* (OTU2), *Clonostachys* spp. (OTU3, OTU4, and OTU9), *Fusarium* spp. (OTU5), *M.*
210 *flavoviride* complex (OTU6), an undetermined Cordycipitaceae genus (OTU7), and *Penicillium* sp. (OTU8) (Fig. 2).
211 For OTU2, DNA sequences corresponded to both *C. cicadae* and *C. lepidopterorum*. However, conidial measurements
212 of isolate S17 (OTU2) were 7.0–9.5 × 2.5–4.0 µm, aligning more closely with *C. cicadae* HKAS 102460 (5.5–9 × 2–
213 3.5 µm) (Wei et al. 2022) than *C. lepidopterorum* TBRC 7263 (9.5–12 × 4.5 µm; Mongkolsamrit et al. 2018).

214 Consequently, isolate S17 was identified as *C. cicadae*. Since other OTU2 strains exhibited conidial sizes and colony
215 morphologies similar to those of S17, all OTU2 strains were classified as *C. cicadae*. Among the eight OTUs, the *M.*
216 *anisopliae* complex (OTU1) had the highest occurrence (52.0%; 13/25), followed by *C. cicadae* (OTU2) (24.0%;
217 6/25) and *Clonostachys* spp. (OTU3) (20.0%; 5/25). Only OTU1 and OTU2 were detected by all five bait insects. The
218 detection rate of the total EPFs was significantly different among the five bait insect ($p = 1.3 \times 10^{-4} < 0.001$). The
219 detection rate of the total EPFs with the *S. litura*-based method (8.0%; 2/25) was the lowest and significantly lower
220 than those with the termite (56.0%; 14/25), mealworm (48%; 12/25), and wax moth (40%; 10/25) (adjusted $p < 0.05$)
221 (Fig. 2). For the detection rate of each OTU, only OTU1 and OTU3 differed among the five bait insect (OTU1: $p =$
222 $4.3 \times 10^{-3} < 0.01$; OTU3: $p = 1.7 \times 10^{-2} < 0.05$). However, no significant differences were found in pairwise comparisons
223 for both OTUs (adjusted $p < 0.05$). The two EPFs detected using *S. litura* as bait were classified as OTU1 (strain S1)
224 and OTU2 (strain S17) (Fig. 2).
225
226 **Characterization and phylogenetic analysis of *C. cicadae* isolated from the soil**
227 In the isolation experiment, a total of 11 *C. cicadae* isolates were obtained from the soil. The morphological
228 characteristics of these isolates were compared with those of *C. cicadae* derived from cicada nymphs for identification.
229 On the five bait insect species, *C. cicadae* developed a thin mycelial layer containing phialides and conidia on the
230 surface of infected cadavers (Fig. 1E, H–K). Numerous short mycelial protrusions were observed on the cadavers of
231 green bottle fly and wax moth larvae; however, these structures differed markedly from the synnemata typically
232 observed on cicada nymphs, such as those of OMNS190829-1 (Fig. 1A). The conidial dimensions of two *C. cicadae*
233 isolates, one from soil (S17) and one from cicada nymphs (OMNS190829-1), were 7.0–9.5 × 2.5–4.0 µm (mean 7.9

× 3.1 µm; n = 20) and 6.0–10.0 × 2.5–4.0 µm (mean 7.9 × 3.3 µm; n = 20), respectively, with no significant differences (two-tailed *t*-test, α = 0.05).

The phylogenetic placement of *C. cicadae* isolated from soil was determined by comparison with *C. cicadae* from cicada nymphs and previously reported *C. cicadae* soil isolates. The ITS region sequences of the 11 *C. cicadae* strains were identical and matched the reference strain *C. cicadae* RCEF HP090724-31, as well as sequences from nine *C. cicadae* isolates from Hemiptera (Cicadellidae), two *C. lepidopterorum* isolates from lepidopteran larvae, and two *C. chanhua* isolates from cicada nymphs. This ITS sequence differed by one base pair from *C. cicadae* HKAS 102460 and by three base pairs from *C. cicadae* strain ORP-9, an isolate obtained from soil using wax moth larvae as bait in Turkey (Şahin and Yanar, 2023). In the phylogenetic analysis of the ITS region, all these strains, except ORP-9, clustered at a terminal node within the *C. cicadae* complex clade, while ORP-9 was positioned as the closest lineage outside the clade. In contrast, three Portuguese *C. cicadae* isolates from the soil using wax moth and mealworm larvae as bait (GMSL29, GMSL114, and TMSL149) identified by Sharma et al. (2018) were placed close to *C. cateniannulata* and distant from the *C. cicadae* complex clade.

Phylogenetic analysis of the three combined loci (LSU, RPB2, and TEF) revealed that the six *C. cicadae* isolates, two from soil (M20 and S17) and four from cicada nymphs (OMNS190829-1, OMNS220717-3, OMNS220811-3, OMNS220911-3), clustered with the three previously reported *C. cicadae* isolates, two *C. lepidopterorum* isolates and two *C. chanhua* isolates (Fig. 3B).

Virulence of EPF isolates toward larvae

253 Virulence against last-instar larvae was compared among six and five representative isolates from OTU1 (*M. anisopliae* complex) and OTU2 (*C. cicadae*), respectively; one isolate from each of the other six OTUs (OTU3, 5–9); and four *C. cicadae* isolates derived from cicada nymphs. Isolates belonging to OTU1 and OTU2 were relatively virulent, but with substantial variability. The maximum mortality caused by OTU1 isolates was 80% (F6), while the minimum was 33% (S1). Similarly, the maximum mortality for OTU2 isolates was 93% (T17.22), and the minimum was 33% (M20, OMNS220811-3). The mortalities caused by the isolates from OTU3 to OTU8 were not significantly higher than the control experiment. For all strains except *Fusarium* sp. T14.20 (OTU5) and *Penicillium* sp. G17 (OTU8), mycelium growth was observed on the corpses, confirming fungal recovery.

261 The LC_{50} values for third-instar larvae, last-instar larvae, and pupae were determined for two highly virulent strains (*M. anisopliae* complex F6 and *C. cicadae* S17). Among the three developmental stages, the LC_{50} values were the lowest for third-instar larvae and highest for pupae for both strains. The LC_{50} values for *M. anisopliae* complex F6 were consistently lower than those for *C. cicadae* S17 across all stages.

265

266 **Discussions**

267 This study revealed several key findings. First, *C. cicadae* was detected from natural soil at a high occurrence, second only to the *M. anisopliae* complex, using a bait method with the common cutworm (*S. litura*) and four other insect species. Second, molecular phylogenetic analysis demonstrated that *C. cicadae* isolates from soil and cicada nymphs belong to the same terminal node, which also includes *C. lepidopterorum*, a species derived from lepidopteran larvae. Finally, the virulence of *C. cicadae* against *S. litura* varied significantly among strains, with some highly virulent strains exhibiting virulence comparable to the *M. anisopliae* complex. In terms of

273 virulence, *C. cicadae* shows comparable potential to the *M. anisopliae* complex for the biological control of *S.*

274 *litura*. These findings highlight the potential application of *C. cicadae* as an effective biocontrol agent, particularly

275 *Cordyceps cicadae* is generally recognized as a specialist pathogen of cicada nymphs. In Japan, *C. cicadae*

276 has been reported to cause epizootics in populations of subterranean nymphs of *Meimuna opalifera* (Hemiptera:

277 Cicadidae) (Isono, 2021). However, several studies have demonstrated that *C. cicadae* also exhibits pathogenicity

278 toward insects beyond cicada nymphs. For instance, *C. cicadae* has been isolated from soil using wax moth larvae

279 (*Galleria mellonella*), mealworm (*Tenebrio molitor*), and peach fruit moth (*Carposina sasakii*) as bait insects (Barker

280 and Barker, 1998; Yaginuma et al. 2002; Sharma et al., 2018; Şahin and Yanar, 2023). Laboratory virulence assays

281 further support the pathogenicity of *C. cicadae* against various lepidopteran larvae. For example, Bugti et al. (2024)

282 reported that *C. cicadae* exhibited virulence against *S. frugiperda* comparable to that of the generalist fungi *Beauveria*

283 *bassiana* and *M. anisopliae*. Similarly, Şahin and Yanar (2021) demonstrated moderate virulence of *C. cicadae* against

284 *S. littoralis*. Yaginuma et al. (2002) found that *C. cicadae* was more virulent against peach fruit moth pupae than

285 *Paecilomyces farinosus* and *P. fumosoroseus*, both of which are generalists with a preference for lepidopterans

286 (Zimmermann, 2008). Additionally, Wang (1988) showed strong virulence of *C. cicadae* against 12 lepidopteran

287 insect species, surpassing the effectiveness of *P. farinosus* and *P. javanicus*. Chen et al. (1990) also documented

288 pathogenicity of *C. cicadae* against the cabbage white butterfly (*Pieris rapae*). Our present study confirms that *C.*

289 *cicadae* can be isolated from soil using five different insect baits. To our knowledge, this is the first report of an

290 entomopathogenic fungus being isolated from soil using *Spodoptera* spp. as bait insects. DNA sequence analysis

291 further confirmed that the ITS sequences of these *C. cicadae* isolates were identical to those derived from cicada

292 nymphs in Japan. These findings suggest that *C. cicadae* possesses pathogenicity toward a wide range of insect hosts.

293 When referring to the previous studies that reported the pathogenicity of *C. cicadae* against lepidopterans,
294 the possibility of misidentification must be considered. Species morphologically similar to *C. cicadae* exist, as noted
295 by Samson et al. (1974) and Mongkolsamrit et al. (2018); however, older studies did not leverage DNA information
296 for species identification. Among the studies mentioned, only Sharma et al. (2018), Şahin and Yanar (2021), and Şahin
297 and Yanar (2023) utilized DNA sequence data for species identification. Here, phylogenetic analysis revealed that the
298 three isolates identified as *C. cicadae* by Sharma et al. (2018) belong to the *C. cateniannulata* clade rather than the *C.*
299 *cicadae* complex (Fig. 3A). They used the DNA sequence of the ITS region of *Isaria cicadae* strain ARSEF 7260
300 (Acc. No.: HQ880826.1) as a reference for *C. cicadae*, however, the species identification of ARSEF 7260 appears to
301 be incorrect as it is placed close to the type strain of *C. cateniannulata* (CBS 152.83) in the phylogenetic analysis of
302 the ITS and consistently distant from the *C. cicadae* complex clade in the phylogenetic analysis of the ITS and the
303 combine dataset (Fig. 3A, B). Additionally, the strain ORP-9, identified as *C. cicadae* by Şahin and Yanar (2021,
304 2023), was placed closest to but outside the *C. cicadae* clade in the ITS phylogenetic analysis (Fig. 3A). Furthermore,
305 the fungal strain FRP42, reported as *C. cicadae* by Yaginuma (2002), had conidia measuring 9.2–11.8 × 2.4–3.6 µm,
306 which was larger than the conidial size of *C. cicadae* HKAS 102460 (5.5–9 × 2–3.5 µm) (Wei et al. 2022) and closer
307 in length to *C. lepidopterorum* (9.5–12 × 4.5 µm) (Mongkolsamrit et al. 2018). *Cordyceps lepidopterorum* is a recently
308 described species and its biology is therefore almost unknown (Mongkolsamrit et al. 2018). Its known host is only
309 lepidopteran larva in Thailand. Only anamorph is known for this species. It was noted that its anamorph was
310 morphologically similar to that of *C. cicadae*, but were distinguished by the size of conidia. Given the complex
311 taxonomic situation of *C. cicadae*, in this study, we conducted a multi-locus phylogenetic analysis. The results
312 revealed that strains S17 and OMNS100829-1, isolated from soil and cicada nymphs respectively, clustered together

313 with reference strains from the *C. cicadae* complex (*C. cicadae*, *C. chanhua*, *C. lepidopterorum*, and *C. jakajanicola*).
314 Morphological examination confirmed that the conidial size of strain S17 was closer to reference strains of *C. cicadae*
315 than to those of *C. lepidopterorum*. However, the classification within the *C. cicadae* complex remains insufficiently
316 supported, even with multi-locus phylogenetic data. Wei et al. (2022) also highlighted that the establishment of *C.*
317 *lepidopterorum* and *C. jakajanicola* lacks robust phylogenetic evidence. Within this species complex, only *C.*
318 *lepidopterorum* is known to target lepidopteran larvae as natural hosts, whereas the other species primarily target
319 cicada nymphs. The strong virulence of *C. cicadae* against lepidopteran larvae suggests potential similarities in
320 pathogenicity, phylogenetic affiliation, and morphology between *C. cicadae* and *C. lepidopterorum*. These complex
321 taxonomic statuses of the *C. cicadae* complex suggest the need for a reconsideration of classification based on
322 additional DNA sequence information as well as ecological and phenotypic information.

323 Despite laboratory evidence from the present and previous studies suggesting that *C. cicadae* has a broader
324 host range, no natural hosts other than cicada nymphs have been reported in the field. Two possible explanations may
325 account for this discrepancy. First, *C. cicadae* may lack the ability to infect insects other than cicada nymphs under
326 natural conditions. While laboratory experiments have demonstrated its physiological capacity to infect diverse insect
327 hosts, *C. cicadae* may not possess other essential traits required for outdoor infection, such as adaptation to the
328 temperature and humidity of the target insect's habitat. The lack of natural infection of *C. cicadae* with *S. litura* may
329 be due to habitat differences; with *C. cicadae* living in forests and *S. litura* in Japan living around farmland (e.g.,
330 Isono, 2021). Alternatively, *C. cicadae* may indeed infect non-cicada hosts underground but remains underreported
331 due to differences in its life cycle. When *C. cicadae* infects cicada nymphs, it forms synnemata that emerge above
332 ground, making it easily detectable in the field (Fig. 1A, B). In contrast, infection of other insects may result in less

333 frequent synnemata formation, as observed in this study with five bait insects (Fig. 1F, J–M), rendering such infections
334 less noticeable in the field.

335 This study demonstrated that *C. cicadae* can be isolated from soil using five different bait insects, including
336 *S. litura*, indicating its potential broad host range. Furthermore, the *C. cicadae* population in Japan exhibits substantial
337 variation in virulence against *S. litura*, with the most virulent strain displaying efficacy comparable to that of the *M.*
338 *anisopliae* complex. However, in order to evaluate its potential as an insecticide, further studies are necessary to assess
339 efficacy in field, field persistence, mass production, formulation, and preservation in the future. The present study did
340 not address these properties. As these properties are largely unknown, the advantages of *C. cicadae* as a biocontrol
341 agent compared to *Beauveria* and *Metarhizium* are also still unknown. With regard to shelf life, *C. cicadae* have been
342 reported to retain their activity after one year of storage at room temperature (Chen et al. 1990). Recent omics study
343 revealed *C. cicadae* has a similar number of protease and hydrolytic enzyme genes compared to M. robertsii and B.
344 bassiana, but fewer biosynthetic gene clusters for secondary metabolites, suggesting that they are different in terms of
345 pathogenicity (Lu et al. 2017). Therefore, the selectivity of *C. cicadae* for harmful and beneficial insects may reveal
346 advantages of *C. cicadae* in biological control (e.g. less impact on beneficial insects). Also, as *C. cicadae* is forest-
347 dwelling, it is expected to have poorer persistence in hot field environments compared to *Beauveria* and *Metarhizium*.
348 The addition of UV protectants such as fluorescent brighteners has been reported to improve field persistence of
349 entomopathogenic fungi (e.g., Inglis et al. 1995), and further development of such techniques would enhance the
350 potential of *C. cicadae*.

351 The present study did not elucidate the mechanisms of varied virulence of the *C. cicadae* isolates. At least
352 no evidence was found that the varied virulence was associated with phylogenetic diversity within a species. These

353 isolates should have variation in virulence factors, but the virulence factors of *C. cicadae* have been little studied.
354 Possible virulence factors for *C. cicadae* against *S. litura* are the secondary metabolites oosporein and beauvericin.
355 These metabolites are virulence factors against lepidopteran larvae in *B. bassiana* and have been found to be produced
356 by *C. cicadae* (Feng et al. 2015; Lu et al. 2017; Xu et al. 2008). In the future, the search for insecticidal molecules and
357 reverse genetics studies using omics data will reveal other virulence factors in *C. cicadae*. Further research on
358 virulence factors of *C. cicadae* will also elucidate the mechanisms of virulence diversification in *C. cicadae*.
359

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370

371 **Declaration**

372 None of the authors has any conflicts of interest or any financial ties to disclose.

373

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517

518

519 **Figure legends**

520

521 **Fig. 1**. *Cordyceps cicadae* isolated from natural cicada nymphs and soil using different bait insects. (A–E) *C.*

522 *cicadae* strain OMNS190829-1 isolated from a natural cicada nymph (*Meimuna opalifera*). (A) Dried specimen of

523 the natural host, (B) Synnemata on the ground, (C) Phialides, (D) Conidia, (E) Colony on PDA (8-days-old), (F–I)

524 *C. cicadae* strain S17 isolated from soil using common cutworm (*Spodoptera litura*, larva) as bait: (F) Infected

525 cadaver of *S. litura*, (G) Phialides, (H) Conidia, (I) Colony on PDA (8-days-old). (J–M) *C. cicadae* detected from

526 soil using different insect baits: (J) Subterranean termite (*Reticulitermes speratus*, worker), (K) Greater wax moth

527 (*Galleria mellonella*, larva), (L) Green bottle fly (*Lucilia sericata*, larva), and (M) Mealworm (*Tenebrio molitor*,

528 larva). Bars: 1 cm (A, B, E, F, I), 10 µm (C, D, G, H), 1 mm (J), 5 mm (K–M).

529

530 **Fig. 2.** Occurrence of *Metarhizium anisopliae* complex and *Cordyceps cicadae* from 25 soil samples using five

531 different insect baits. Difference of the occurrence among the five insect baits was tested by Cochran's Q test. The

532 resulted *p*-values were presented over the bars. The different letters on the bars indicate significant differences

533 within each fungal group on the x-axis (McNemar test, adjusted $p < 0.05$).

534

535 **Fig. 3.** A maximum likelihood phylogenetic tree inferred from DNA sequences of (A) ITS1-5.8S rDNA-ITS2 and (B)

536 a concatenated dataset (LSU, RPB2, and TEF) of *Cordyceps cicadae* and its closely related species. Host information

537 symbols are placed on the labels of the *C. cicadae* complex. Accession numbers for each DNA sequence are shown

538 at the beginning of the labels in the ITS phylogeny. The analysis of ITS was performed to clarify the molecular

539 phylogenetic relationships among the soil-derived and cicada-derived *C. cicadae* isolates from the present and
540 previous studies. The analysis of the combined three loci was performed to clarify the relationship among the *C.*
541 *cicadae* isolates in the present study and the *C. cicadae* complex members.
542
543 **Fig. 4.** Comparison of the virulence of 17 representative fungal isolates from eight OTUs and four *C. cicadae* isolates
544 derived from natural cicada nymphs against *Spodoptera litura.* Bars and error bars indicate mean of mortality and
545 standard error (n=3). Bars with the same letters are not significantly different (Tukey's HSD test, $\alpha = 0.05$).
546
547 **Fig. 5.** Virulence of *Cordyceps cicadae* strain S17 and *Metarhizium anisopliae* complex strain F6 against three
548 developmental stages of the common cutworm (*Spodoptera litura*). Error bars indicate 95% confidence intervals.
549 The virulence of the two strains with high virulence against the last-instar larvae of *S. litura* was assessed by the
550 LC_{50} value against the three developmental stages.

Supplementary Table 1. All strains isolated by bait method from soil samples collected in Kyushu Univ. Ito campus, Fukuoka, Japan.

Soil Sample	Isolates[1]	OTU	Bait insects	Accession No. for rDNA ITS
1	T1.16	1	Termite	LC849550
	G1	1	Wax moth	LC849549
	M1	1	Mealworm	LC849546
	S1	1	Cutworm	LC849559
2	T2.2	1	Termite	LC849563
	M2	4	Mealworm	LC849535
3	G3a	1	Wax moth	LC849552
	G3b	2	Wax moth	LC849522
4	M4	1	Mealworm	LC849547
	F4	1	Fly	LC849541
	T4.1	2	Termite	LC849528
	G4	2	Wax moth	LC849523
	G4a	2	Wax moth	LC849524
5	M5	3	Mealworm	LC849568
6	T6.8	1	Termite	LC849544
	T6.17	1	Termite	LC849545
	G6	1	Wax moth	LC849543
	F6	1	Fly	LC849542
7	T7.15	1	Termite	LC849560
	F7	2	Fly	LC849521
8	T8.20	3	Termite	LC849565
11	T11.11	1	Termite	LC849554
	G11	1	Wax moth	LC849553
	M11	3	Mealworm	LC849569
12	T12.18	1	Termite	LC849551
	G12	7	Wax moth	LC849539
14	T14.20	5	Termite	LC849537
	M14	5	Mealworm	LC849538
15	G15m	1	Wax moth	LC849556
	G15b	2	Wax moth	LC849525
16	M16	5	Mealworm	LC849536
17	T17.2	2	Termite	LC849529
	T17.12	2	Termite	LC849530
	T17.22	2	Termite	LC849531

	S17	2	Cutworm	LC849527
	G17	8	Wax moth	LC849540
	M17	4	Mealworm	LC849534
19	M19	3	Mealworm	LC849567
	T19.22	6	Termite	LC849564
20	T20.23	1	Termite	LC849548
	G20	1	Wax moth	LC849557
	M20	2	Mealworm	LC849526
21	T21.6	1	Termite	LC849561
	G21	1	Wax moth	LC849558
	M21	3	Mealworm	LC849566
22	T22.4	1	Termite	LC849562
23	M23	4	Mealworm	LC849532
24	M24	4	Mealworm	LC849533
25	M25	1	Mealworm	LC849555

[1] The first alphabet letter of the fungal isolates names indicates the type of bait insect (i.e., F: Green bottle fly; G: greater wax moth; M: mealworm; T: termite, S: common cutworm *S. litura*) and the number following it indicates the soil sample number.

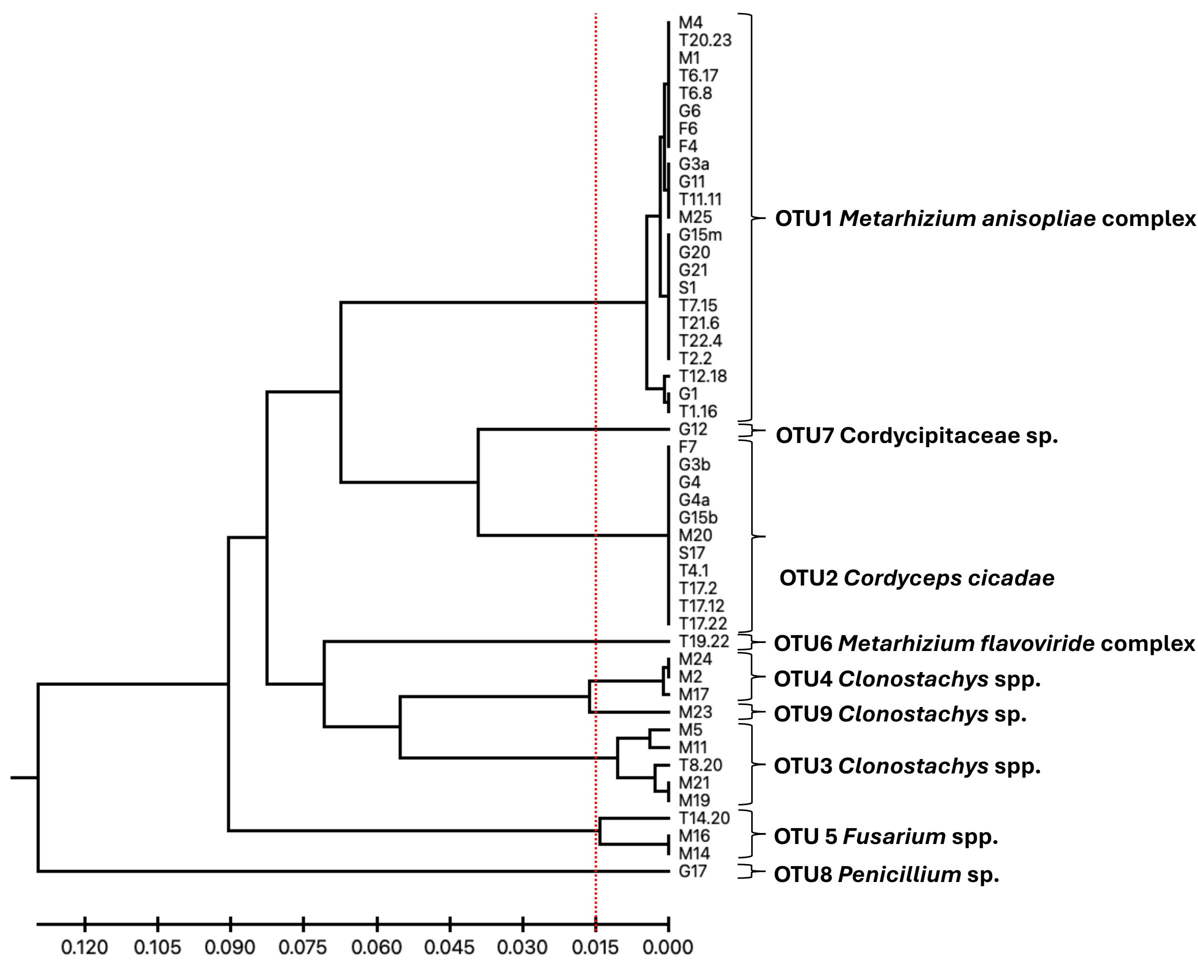
Supplementary Table 2. Primers and PCR conditions

Loci	Primers for PCR and sequencing (5'-3')	References	PCR condition
ITS	ITS5 (GGAAGTAAAAGTCGTAACAAGG) ITS4 (TCCTCCGCTTATTGATATGC)	White et al. (1990)	38 cycles of 98˚C 10 s, 55˚C 5 s, 68˚C 7 s
LSU	LR0R (ACCCGCTGAACTTAAGC) LR5 (TCCTGAGGGAAACTTCG)	Vilgalys and Hester (1990)	38 cycles of 98˚C 10 s, 55˚C 5 s, 68˚C 10 s
RPB2	fRPB2-5F2 (GGGGWGAYCAGAAGAAGGC) fRPB2-7cR (CCCATRGCTTGYTTRCCCAT)	Castlebury et al. (2004)	38 cycles of 98˚C 10 s, 55˚C 5 s, 68˚C 10 s
TEF	983F (GCYCCYGGHCAYCGTGAYTTYAT) 2218R (ATGACACCRACRGCRACRGTYTG)	Rehner and Buckley (2005)	38 cycles of 98˚C 10 s, 55˚C 5 s, 68˚C 10 s

Supplementary table 3. Information for taxa used for phylogenetic analysis in this study.

Species	Strain	Isolation source	ITS	LSU	TEF	RPB2	Reference
Cordyceps blackwelliae	TBRC 7255	Coleoptera (larva)	MF140737.1	MF140703.1	MF140823.1	MF140796.1	Mongkolsamrit et al. (2018)
	TBRC 7256	Coleoptera (larva)	NR_164416.1	NG_067805.1	MF140822.1	MF140795.1	Mongkolsamrit et al. (2018)
Cordyceps bifusispora	EFCC 5690	Lepidopteran (pupa)		EF468806.1	EF468746.1	EF468909.1	Sung et al. (2007)
	ARSEF 5690	Lepidopteran (pupa)	AY245627.1				Li et al. (2008)
Cordyceps cateniannulata	CBS 152.83^{T}	Coleoptera (adult)	AY624172.1	MG665226.1	JQ425687.1	MG665236.1	Luangsa-ard et al. (2005)
	TBRC 7258	Araneae; spider	MF140753 .1				Mongkolsamrit et al. (2018)
Cordyceps chanhua	JGS150713-1	Hemiptera: Cicadidae (nymph, *Platylomia pieli*)	MT192488.1	MT239107.1	MT268246.1	MT268244.1	Li et al. (2021)
	BA-001		MT555325.1	MT555409.1	MT637810.1	MT637807.1	Li et al. (2021)
C. cicadae	ARSEF 7260	Hymenoptera	HQ880826.1		HQ881017.1	HQ880970.1	Rehner et al. (2011)
	BCMU CS03	Hemiptera: Cicadidae (nymph, *Graptopsaltria nigrofuscata*)	AB085888.1				Yokoyama et al. (2004)
	BCMU CS02	Hemiptera: Cicadidae (nymph, *Platypleura kaempferi*)	AB085887.1				Yokoyama et al. (2004)
	HKAS:102460	Hemiptera: Cicadidae (nymph)	OQ127350.1	OQ127384.1	OQ186376.1	OQ186402.1	Wei et al (2022)
	GMSL29	Soil (bait with a wax moth larva)	LT220699.1				Sharma et al. (2018)
	GMSL114	Soil (bait with a wax moth larva)	T220698.1				Sharma et al. (2018)
	TMSL149	Soil (bait with a mealworm larva)	T220700.1				Sharma et al. (2018)
	ORP-9	Soil (bait with a wax moth larva)	MW410194.2				Şahin and Yanar (2021)
	OMNS190829-1	**Hemiptera: Cicadidae**	**LC849519.1**	**LC847112.1**	**LC847116.1**	**LC847114.1**	**This study**
	OMNS220717-3	**Hemiptera: Cicadidae**	**LC849517.1**	**LC876966.1**	**LC876974.1**	**LC876970.1**	**This study**
	OMNS220811-3	**Hemiptera: Cicadidae**	**LC849518.1**	**LC876967.1**	**LC876975.1**	**LC876971.1**	**This study**
	OMNS220911-3	**Hemiptera: Cicadidae**	**LC849520.1**	**LC876968.1**	**LC876976.1**	**LC876972.1**	**This study**
	M20	**Soil (bait with a mealworm)**	**LC849526.1**	**LC876965.1**	**LC876973.1**	**LC876969.1**	**This study**
	S17	**Soil (bait with a cutworm)**	**LC849527.1**	**LC847113.1**	**LC847117.1**	**LC847115.1**	**This study**
	RCEF HP090724-31	Hemiptera: Cicadidae	AF368801.1	MF416552.1	MF416496.1	MF416447.1	Kepler et al. (2017)
Cordyceps lepidopterorum	TBRC 7263	Lepidoptera (larva)	NR_164422.1	NG_067804.1	MF140819.1	MF140792.1	Mongkolsamrit et al. (2018)
	TBRC 7264	Lepidoptera (larva)	MF140766.1	MF140700.1	MF140820.1	MF140793.1	Mongkolsamrit et al. (2018)
Cordyceps jakajanicola	BCC 79816	Hemiptera: Cicadidae		MN275696	MN338479	MN338489	Crous et al. (2019)
	BCC 79817	Hemiptera: Cicadidae		MN275697	MN338480	MN338490	Crous et al. (2019)

Cordyceps qingchengensis	MFLU 17-1022	Lepidoptera; Bombycidae	KY423506.1	MK761211	MK770630		Zha et al. (2019)
	HKAS:102444	Insect cocoon in soil	OQ127352.1	OQ127386.1	OQ186378.1	OQ186403.1	Wei et al (2022)
Cordyceps tenuipes	TBRC 7265	Lepidopteran (pupa)	MF140741.1	MF140707.1	MF140827.1	MF140800.1	Mongkolsamrit et al. (2018)
Cordyceps pseudotenuipes	YFCC 8404	Lepidoptera		NG_149014.1	OL473527.1	OL473538.1	Dong et al. (2022)
Cordyceps subtenuipes	YFCC 6051			NG_079651.1	MN576945.1	MN576891.1	Wang et al. (2020)
Cordyceps simaoensis	YFCC 8406	Lepidoptera		NG_149015.1	OL473529.1	OL473540.1	Dong et al. (2022)
Cordyceps spegazzinii	CG151	–	OP146349.1				–
Cordyceps farinosa	CBS 111113[T]	–	AY624181.1				Luangsa-ard et al. (2005)
Cordyceps fumosorosea	CBS:244.31	Butter	AY624182.1	MG665230.1	JQ425690.1	MF416454.1	Kepler et al. (2017) Luangsa-ard et al. (2005)
Cordyceps morakotii	TBRC 7276 [T]	Hymenoptera; ant (pupa)	KT261390.1				Tasanathai et al. (2016)
Cordyceps polyarthra	CG151	–	OP146350.1				–
Cordyceps pruinosa	ARSEF 5413	Lepidoptera; *Iragoides fasciata*	JN049826 .1				Kepler et al. (2012)
Cordyceps sp.	NBRC 106954	Hemiptera: Cicadidae (nymph , *Meimuna opalifera*)	AB916360.1				Grudniewska et al. (2014)



Supplementary Figure 1. UPGMA clustering of DNA sequence of ITS1-5.8S rDNA-ITS2 from the 49 fungal soil isolates. OTU grouping was performed with *p*-distance=0.03 as the threshold