ENTOMOPATHOGENIC FUNGI (*Cordyceps* spp.) ISOLATE FROM AMERGING INSECT PESTS ASSOCIATED WITH PLANTATION FORESTS IN VIET NAM

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ABSTRACT

Plantation forests accounted for 4.4 million *hectares* out of 14.7 million *hectares* of total forest area in Vietnam and contribute greatly to the export of timber and wood products as well as the livelihoods of the mountainous people. Several species of insect pests have been observed in *Dendrocalamus barbatus*, *Chukrasia tabularis*, *Acacia* and *Eucalyptus* plantations. However, these pests were found to be parasitized by entomopathogenic fungi. The aims of this study were to examine the pathogenicity and identify the taxonomy of entomopathogens obtained from *Ceracris kiangsu*, *Episparis tortuosalis*, *Batocera lineolata* and *Endoclita* sp. Results showed that seven isolates of the entomopathogenic fungi were found from these four pests. In addition, all identified isolates expressed pathogenicity in *Galleria mellonella* larvae with the parasitism range was from 73 to 100%. Based on ITS, LSU and TEF1-α sequence analysis, the identified entomopathogenic fungi belong to the genus *Cordyceps*. This finding suggests a great potential for application of these *Cordyceps* isolates in insect pest management programs in Vietnam.

Keywords: *Batocera lineolata, Ceracris kiangsu, Endoclita sp., entomopathogenic fungi, Episparis tortuosalis.*

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1. INTRODUCTION

Vietnam has about 4.4 million hectares of planted forests comprised of acacias and eucalypts, which are the exotic species with an estimated area of 2,000,000 and 400,000 ha, respectively [1]. In addition, *Dendrocalamus barbatus* and *Chukrasia tabularis* are native species grown widely with an estimated area of 120,000 and 35,000 hectares [1]. In recent years, forest tree species are frequently attacked by different folivores and stem borers which have been identified as *Ceracris kiangsu* Tsai (Orthoptera: Acrididae) in *D. barbatus* [2], *Episparis tortuosalis* Moore (Lepidoptera: Erebidae) in *C. tabularis* [3], *Batocera lineolata* Chevrolat (Coleoptera: Cerambycidae) in *Eucalyptus* hybrid (*E. urophylla* × *E. grandis*) [4] and *Endoclita* sp. in *Acacia* and *Eucalyptus* plantations (Unpublished data). However, these insect pests were found to be parasitized by the entomopathogenic *Cordyceps* spp., with the rate of 10-15% [2, 5].

Various species of entomopathogenic fungi have been isolated and identified from parasitized insects such as *Beauveria bassiana* from *Cephalcia*

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tannourinensis [6], *B. lii* from *Henosepilachna vigintioctopunctata* [7], and *B. sinensis* from Geometridae (Lepidoptera) [8]. *Cordyceps javanica* and *C. fumosorosea* from *Bemisia tabaci* [9] and *Diaphorina citri* [10]. Several entomopathogens have been used effectively in the biological control of different insect pests [11, 12, 13]. For example, *B. bassiana* and some species of the genus *Cordyceps* were used as microbial insecticides [14, 15]. Because the use of biological agents has been reduced the use of chemical pesticides [12, 13]. Management strategies for plantation diseases and insect pests in Vietnam is also prioritized towards the approach of integrated pest management (IPM), especially using biological agents [16]. This paper aimed at pathogenicity assessment and identification of *Cordyceps* spp. isolated from *C. kiangsu*, *E. tortuosalis*, *B. lineolata* and *Endoclita* sp.

2. MATERIALS AND METHODS

2.1. Sampling, isolation, and culture

In June 2021, seven insect samples of *Batocera lineolata* (1 individual), *Endoclita* sp. (2 individuals), *Episparis tortuosalis* (2 individuals) and *Ceracris kiangsu* (2 individuals) parasitized at the larval, pupal and nymph stages were collected in *Acacia mangium*, *Eucalyptus* hybrid, *Dendrocalamus barbatus*, *Chukrasia tabularis* plantations in Hoa Binh, Phu Tho, Thanh Hoa and Nghe An provinces (Table 1).

Table 1. Entomopathogens obtained from developmental stages of insect pests at different sites

Sample	Insect pest	Stage	Plantation	Location
B2	*Endoclita* sp.	Larva	*Acacia mangium*	Tan Lac, Hoa Binh
B3	*Endoclita* sp.	Larva	*Eucalyptus* hybrid	Yen Lap, Phu Tho
B4	*Batocera lineolata*	Pupa	*Eucalyptus* hybrid	Yen Lap, Phu Tho
B5	*Ceracris kiangsu*	Nymph	*Dendrocalamus barbatus*	Muong Lat, Thanh Hoa
B6	*Ceracris kiangsu*	Nymph	*Dendrocalamus barbatus*	Muong Lat, Thanh Hoa
B7	*Episparis tortuosalis*	Larva	*Chukrasia tabularis*	Nghia Dan, Nghe An
B8	*Episparis tortuosalis*	Larva	*Chukrasia tabularis*	Nghia Dan, Nghe An

Conidia from the surface of each parasitized sample were transferred to Potato Dextrose Agar (PDA) medium in Petri dishes with diameter of 9 cm, and incubated at 25-26°C in a laboratory. After 5-6 days, when cultures had grown 0.5-1.0 cm in diameter, hyphal tips were subcultured onto new dishes and incubated at 25-26°C. All isolates were stored at the Forest Protection Research Centre, Vietnamese Academy of Forest Sciences.

2.2. Pathogenicity tests on larval *Galleria mellonella*

Initial test was carried out to examine the efficacy of seven entomopathogenic fungal isolates on the 4th larval instars of *Galleria mellonella*

fowllowing the method of Hussein *et al.* (2012) [17]. Conidia of the 14 day-old fungal cultures grown on PDA at 25°C were harvested by scraping the sporulating colonies and suspending in distilled water. The conidial suspension was filtered through two layers of cheesecloth. The spore solution was diluted at a density of about 5×10^6 CFU/ml + 1% Tween 80. *Beauveria bassiana* (Muskardin, dosage of 3.75 g/l) + 1% Tween 80 was applied in the test as a positive control. Steriled water + 1% Tween 80 were also included as negative control. The larvae were submersed for 5s in each treatment. In other test, the treatments were sprayed on the larvae at a dosage of 3 ml/treatment.

In each test, four replicates of each treatment were carried out using fifteen larvae per replicate. After treatment, the larvae were placed in a sterilized moist chamber consisting of a plastic box (9 cm in diameter, 3 cm in depth) with wet filter paper, and incubated at 25-26°C. The number of parasitized larvae were counted after two, three, four days of the direct inoculation and three, six, nine days of the spraying.

2.3. Sequencing and phylogenetic analysis

The primers ITS1/ITS4, 5.8SR/LR7 and EF983F/EF2212R were used to identify the entomopathogenic fungal isolates through ITS, LSU and TEF1-α gene amplification. Amplifications were carried out in 49-µl-volume reactions containing 20 µl Master Mix (Eppendorf, Germany), 1 µl of each forward and reverse primer, 1 µl of DNA template and 27 µl sterilized water. The PCRs were performed with a C1000 Touch™ thermal cycler (Bio-Rad, USA). The PCR cycling parameters were as follows: initial denaturation for 3 minutes at 94˚C, followed by 30 cycles at 94˚C for 30 seconds, 52˚C for 30 seconds and 72˚C for 1 minute. The amplification was completed at 72˚C for 10 minutes and then the PCR product was stored at 10˚C. The PCR amplicons were sequenced at 1st BASE (Malaysia). The DNA sequences were compared to the GenBank database via the nucleotide-nucleotide BLAST search interface located at the National Center for Biotechnology Information, Bethesda, USA. Relevant sequences were transferred and then processed using BioEdit software [18].

Phylogenetic analyses were perfomed using the ITS, LSU and TEF1-α sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [19]. Consensus trees with the highest log likelihoods (1158.71) were created. Evolutionary analyses were conducted in MEGA7 [20]. *Ophiocordyceps sinensis* was used as the outgroup taxon to root the tree.

2.4. Data analysis

The parasitized rate (*P%*) was calculated according to the following equation:

$$P\% = (n/N) \times 100$$

Where: *n* is the number of parasitized insects; *N* is total number of insects assessed.

Data were analysed using GenStat Release 12.1 software package. Analysis of variance was used to test for significant effect of blocks and treatments, followed by Duncan's Multiple Range Test for comparisons of means of different treatments.

3. RESULTS AND DISCUSSION

3.1. Isolation

Parasitized *C. kiangsu* nymphs and *E. tortuosalis* larvae were commonly observed on ground arround the tree crown projection of *D. barbatus* or *C. tabularis,* where humidity is relatively high (Figure 1c). Parasitized *Batocera lineolata* pupa (Figure 1a) and *Endoclita* sp. larvae (Figure 1b) were found in their breeding galleries inside the stem of *Acacia* and *Eucalyptus* trees. Their bodies were dry and covered with numerous white conidia.

Seven entomopathogenic fungal isolates were found in the parasitized insect samples (Table 1) including *Endoclita* sp. (2 isolates), *B. lineolata* (1 isolate), *C. kiangsu* (2 isolates) and *E. tortuosalis* (2 isolates).

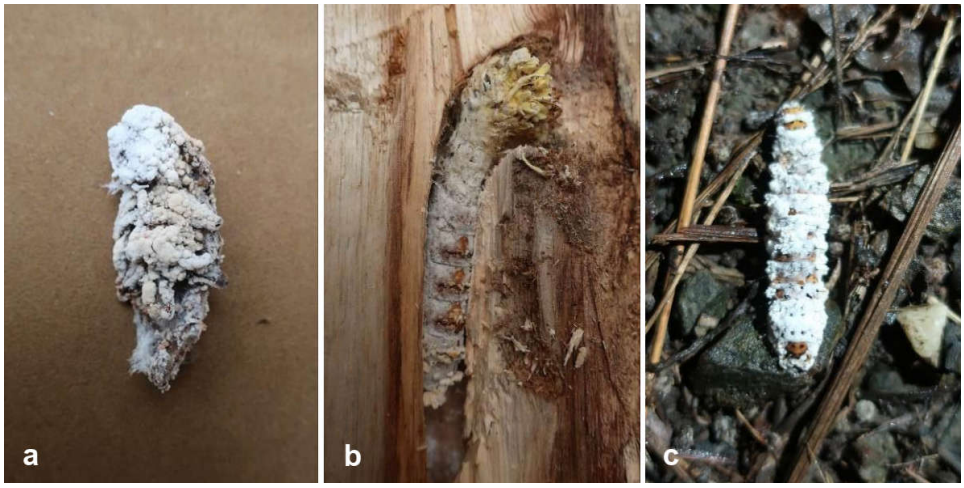

Figure 1. Parasitized insects in forests: a. Pupa of *Batocera lineolata*, b. Larva of *Endoclita* sp., c. Larva of *Episparis tortuosalis*

3.2. Pathogenicity on larval *Galleria mellonella*

There was no significant difference between blocks ($P > 0.05$) in the direct inoculation test and the spraying test (data was not shown). All tested entomopathogenic isolates had pathogenicity on *G. mellonella* larvae (Table 2). The percentage of larvae parasitized by seven isolates (B2, B3, B4, B5, B6, B7 and B8) in the first test method of direct inoculation after two and three days were 51.7-65.0% and 63.3-76.7%, respectively. After four days, the percentage of parasitized larvae reached 85-100%. Three isolates (B6, B7 and B8) infected and covered the whole larvae, similar to the Muskadin (positive control).

Table 2. Percentage of *Galleria mellonella* larvae parasitized in two infection methods

Treatment	Days after direct inoculation (%)			Days after spraying (%)		
	2	3	4	3	6	9
B2	51.7 ± 1.6^{b}	63.3 ± 1.7^{b}	85.0 ± 2.6^{b}	26.7 ± 0.6^{b}	53.3 ± 2.2^{b}	73.3 ± 2.7^{b}
B3	60.0 ± 2.1^{cde}	71.7 ± 2.4^{cd}	93.3 ± 3.1^{c}	30.0 ± 0.9^{b}	63.3 ± 1.8^{cd}	76.7 ± 2.9^{bc}
B4	55.0 ± 1.3^{bc}	65.0 ± 1.7^{bc}	86.7 ± 2.2^{b}	25.0 ± 1.2^{b}	55.0 ± 2.4^{bc}	76.7 ± 3.0^{bc}
B5	60.0 ± 3.2^{cde}	70.0 ± 2.9^{bcd}	93.3 ± 4.1^{c}	26.7 ± 1.7^{b}	61.7 ± 3.3^{bcd}	78.3 ± 3.6^{bc}
B6	61.7 ± 3.5^{cde}	75.0 ± 3.4^{d}	100^{d}	38.3 ± 1.9^{bc}	65.0 ± 2.7^{d}	80.0 ± 2.8^{bc}
B7	65.0 ± 2.8^{e}	76.7 ± 3.8^{d}	100^{d}	50.0 ± 1.6^{c}	68.3 ± 2.4^{d}	81.7 ± 3.5^{c}
B8	63.3 ± 1.9^{de}	75.0 ± 4.5^{d}	100^{d}	41.7 ± 1.3^{bc}	66.7 ± 2.6^{d}	80.0 ± 3.0^{bc}

Treatment	Days after direct inoculation (%)			Days after spraying (%)		
	2	3	4	3	6	9
Muskardin	56.7±1.6^{bcd}	73.3±4.2^{d}	100^{d}	26.7±1.5^{b}	60.0±4.2^{bcd}	76.7±4.3^{bc}
Water	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Notes. Values identified by the same letters in a column are not significantly different among treatments at the 0.05 level according to Duncan's multiple range tests

The percentage of parasitized larvae by seven isolates (B2, B3, B4, B5, B6, B7 and B8) after spraying three, six and nine days were 25-50%, 53.3-68.3% and 73.3-81.7%, respectively. The parasitic effect of the entomopathogenic fungi was slower in the spraying test than the direct inoculation test. After nine days, the percentage of parasitized larvae by the spraying (Figure 2c) was higher in the isolate B7 than the Muskadin (Figure 2d).

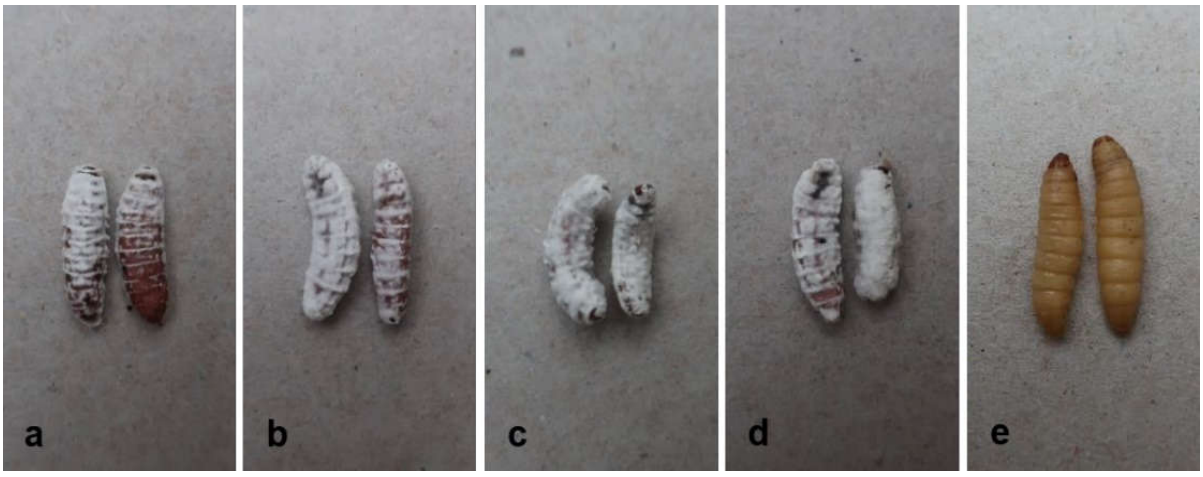

Figure 2. Parasitized *Galleria mellonella* larvae after nine inoculation days: a-d. different isolates; e. controls; a. isolate B2; b. isolate B3; c. isolate B7; d. Muskardin; e. water

3.3. Identification

The ITS, LSU, and TEF1-α gene sequences of seven isolates in this study were compared with reference sequences obtaned from the National Center for Biotechnology (NCBI) GenBank for *Cordyceps bifusispora*, *C. blackwelliae*, *C. chiangdaoensis*, *C. cicadae*, *C. coleopterora*, *C. farinose*, *C. fumosorosea*, *C. ghanensis*, *C. jakajanicola*, *C. lepidopterorum*, *C. militaris*, *C. morakotii*, *C. ninchukispora*, *C. pruinosa*, *C. rosea* and *C. tenuipes* (Table 3). Bootstrap values were equal to or greater than 50% derived from 1000 iterations.

The isolate B2 and B3 did not match to the data available on the NCBI. Five isolates including B4, B5, B6, B7, and B8 was only 98% identical to the *Cordyceps jakajanicola* (Figure 3). In addition, the morphological and sporal characteristics of these isolates are not similar to those of *Coryceps* species that have been recorded previously. These isolates are probably new species and further studies are required to clarify this point.

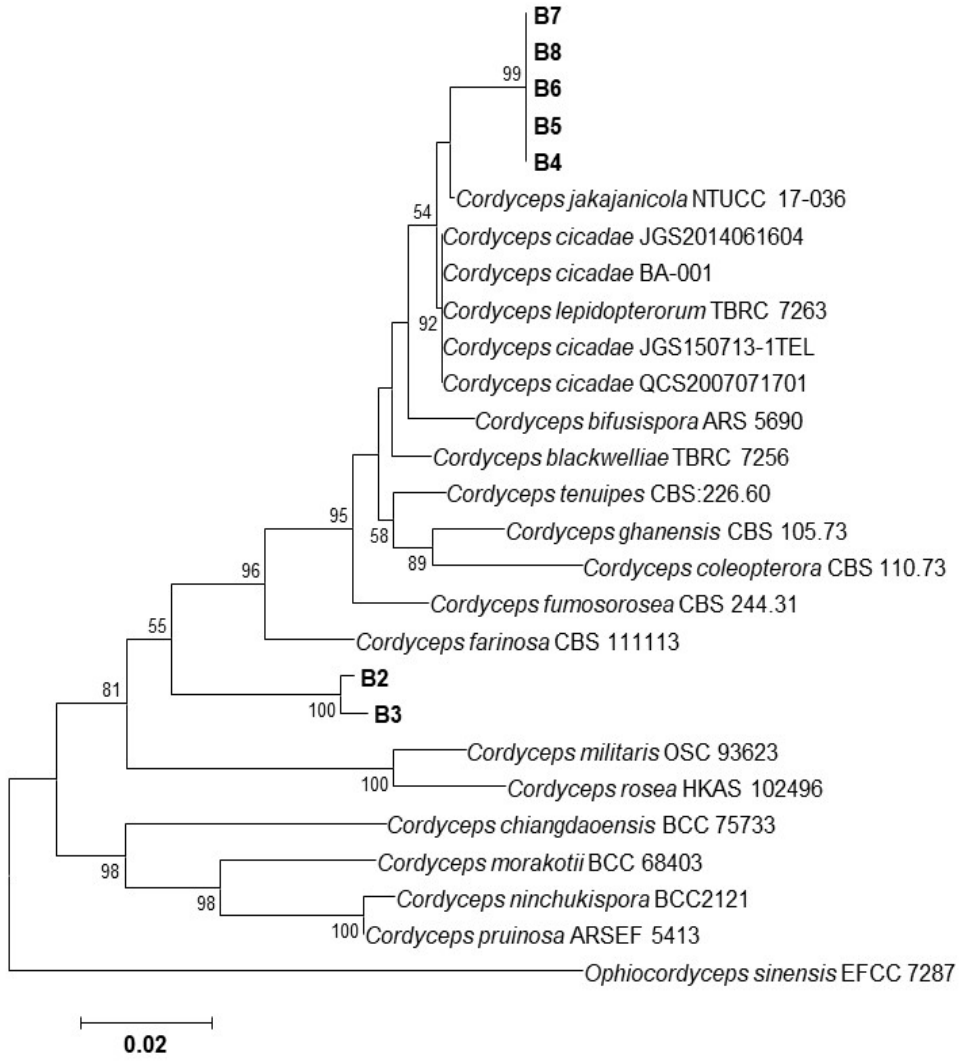

Figure 3. Consensus tree of the concatenated sequences of ITS, LSU and TEF1-α from different species of the genus *Cordyceps.* The Maximum Likelihood method was used to construct the tree. The bar represents an expected sequence variation of 2.0%. *Ophiocordyceps sinensis* was used as the outgroup taxa to root the tree

Table 3. GenBank accession numbers of *Cordyceps* isolates in this study

Species	Isolate	ITS	LSU	TEF1-α
Cordyceps sp.	B2	ON876733	ON876740	ON815106
Cordyceps sp.	B3	ON876734	ON876741	ON815107
Cordyceps sp.	B4	ON876735	ON876742	ON815108

Species	Isolate	ITS	LSU	TEF1-α
Cordyceps sp.	B5	ON876736	ON876743	ON815109
Cordyceps sp.	B6	ON876737	ON876744	ON815110
Cordyceps sp.	B7	ON876738	ON876745	ON815111
Cordyceps sp.	B8	ON876739	ON876746	ON815112
C. bifusispora	ARS 5690	AY245627	EF468806	EF468746
C. blackwelliae	TBRC 7256	NR_164416	MF140702	MF140822
C. chiangdaoensis	BCC 75733	KT261397	MZ573231	KT261407
C. cicadae	JGS2014061604	KX017276	MT239107	MK770632
C. cicadae	BA-001	AEIW01001963	AEIW01001963	AEIW01000182
C. cicadae	JGS150713-1TEL	MT192488	MT239107	MT637809
C. cicadae	QCS2007071701	KX017277	MK761212	MK770631
C. coleopterora	CBS 110.73	AY624177	JF415988	JQ425689
C. farinose	CBS 111113	AY624181	MF416554	MF416499
C. fumosorosea	CBS 244.31	AY624182	MF416557	MF416503
C. ghanensis	CBS 105.73	NR_111171	MH872340	MN338483
C. jakajanicola	NTUCC 17-036	MT966043	MT974255	MW025836
C. lepidopterorum	TBRC 7263	MF140765	MF140699	MF140819
C. militaris	OSC 93623	JN049825	AY184966	DQ522332
C. morakotii	BCC 68403	KT261392	MZ573234	KT261402
C. ninchukispora	BCC 2121	FJ765277	FJ765245	FJ765261

Species	Isolate	ITS	LSU	TEF1-α
C. pruinosa	ARSEF 5413	JN049826	AY184968	DQ522351
C. rosea	HKAS 102496	MT012344	MT012351	MT025050
C. tenuipes	CBS:226.60	MH857959	LUFF01000002	LUFF0100007

3.4. Discussion

This study is the first report of the *Cordyceps* parasitic entomopathogens found in *Batocera lineolata*, *Ceracris kiangsu*, *Endoclita* sp. and *Episparis tortuosalis* in Vietnam. Many parasitic fungi species have been found in insect pests such as *B. bassiana* from *Cephalcia tannourinensis* [6], *Cordyceps javanica* and *C. fumosorosea* from *Bemisia tabaci* [9], *Diaphorina citri* [10], and *B. lineolata* [21]. *C. wuyishanensis* and *C. maolanoides* from cicada and dung beetles [22]. *Ophiocordyceps macroacicularis* and *O. ramosissimum* from *E. davidi* [23].

Different species of *Cordyceps* spp. have not only high medicinal roles [14, 23], but also effective biopesticides [14, 15, 21]. Some entomopathogenic fungi are effective to insect pests [11, 12, 13]. In Switzerland, *B. brongniartii* infection rates of *Melolontha melolontha* was 59-86% [24], and larvae survived in the soil after 16 months of the fungal application [25]. *B. bassiana* and *B. brongniartii* have been shown to be safe entomopathogenic fungi [13]. *B. bassiana* strains 147 were used as an insecticide [11]. *B. brongniartii* and *B. bassiana* were used for the control of *Brahmina coriacea* with the dead larvae rate ranged from 40 to 83% [26]. *Metarhizium anisopliae* and *Isaria fumosorosea* [15] and *C. fumosorosea* [27] were used for the management of *D. citri* in the field. *C. cateniannulata* were used to cotrol of *Spodoptera frugiperdain* in China [28].

Seven *Cordyceps* isolates observed from the parasitized samples of *Endoclita* sp., *B. lineolata*, *C. kiangsu*, and *E. tortuosalis* in this study had high effectiveness when being inoculated into *Galleria mellonella* larvae. The percentage of parasitized *G. mellonella* larvae in the direct inoculation and spraying experiment were 73.3-81.7%, and 85-100%, respectively. *C. bassiana* had a high effective against the nymphs and adults of *D. citri* in the field [15]. *Cordyceps fumosorosea* was used for management of *Bemisia tabaci* [29, 9]. *C. javanica* was used for management of *Diaphorina citri* in the field with effectiveness of 60-90% [30, 10], and *B. lineolata* in China [21].

The *Cordyceps* isolates identified in this study would be potential for management plans of insect pests in Vietnam. Although these isolates were sequenced by the ITS, LSU, and TEF1-α genes, but other primers are recommended for specific identification of the enthopathogenic fungal species.

4. CONCLUSION

Seven entomopathogenic fungal isolates were isolated from *Endoclita* sp. (2 isolates), *B. lineolata* (1 isolate), *C. kiangsu* (2 isolates) and *E. tortuosalis* (2 isolates). These seven isolates were pathogenic to *G. mellonella* larvae with the percentage of parasitism ranged from 73.3 to 100% in both spraying and direct inoculation methods. Based on sequence analysis of ITS, LSU and TEF1-α, all seven entomopathogenic fungi were identified to be *Cordyceps* spp.

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