Vietnam Journal of Biotechnology **20**(2): 317-327, 2022

THE FIRST RECORD OF *METACORDYCEPS NEOGUNNII* (*METACORDYCEPS*, CLAVICIPITACEAE) ISOLATED FROM LARVA OF *LEPIDOPTERA* IN VIETNAM: MORPHOLOGICAL, PHYLOGENETIC CHARACTERIZATION AND CHEMICAL CONSTITUENT ANALYSIS

Lao Duc Thuan[1], Le Huyen Ai Thuy[1], Le Cong Hac[1], Nguyen Hoang Mai[2], Nguyen Van Giang[3], Truong Binh Nguyen[2,✉]

[1]*Faculty of Biotechnology, Ho Chi Minh City Open University, 97 Vo Van Tan Street, Ward 6, District 3, Ho Chi Minh City, Vietnam*
[2]*Institute of Research & Hi-Tech Application in Agriculture, Dalat University, 1 Phu Dong Thien Vuong Street, Da Lat City, Lamdong Province, Vietnam*
[3]*Faculty of Biology, Dalat University, 1 Phu Dong Thien Vuong Street, Da Lat City, Lamdong Province, Vietnam*

✉To whom correspondence should be addressed. E-mail: nguyentb@dlu.edu.vn

Received: 27.8.2021
Accepted: 06.10.2021

SUMMARY

An entomopathogenic fungus, specimen DL0091 parasitized on the larvae of *Lepidoptera*, was collected from Lang Biang Biosphere Reserve, located in Lam Dong Province, Vietnam. The specimen DL0091 has been analyzed to be contained numerous chemical constituents, especially containing adenosine of 634 mg/Kg and cordycepin of 35.2 mg/Kg. Due to containing many bioactive compounds, DL0091 was promised to be a precious natural source that could be applied in fields of medicine and function food for health care. For classification, based on the morphology analysis, it was identified as *Metacordyceps neogunnii* (*Metacordyceps*, *Clavicipitaceae*) sharing the similar characteristics of *M. neogunnii* T.C. Wen & K.D. Hyde. Morphology of this species differed from *Cordyceps neogunnii* (Berk.) Berk., by many characteristics, such as the larger stroma of DL0091 (15–130 mm x 2–6 mm), of asci (550–680 μm × 5–8 μm), etc. Additionally, the combined multi-gene phylogenetic analysis, including *ITS*, *Tef* and *Rpb1*, well supported its systematic position in the clade of *M. neogunnii*, which was used as traditional herb in China and other Asian countries. In summary, DL0091 was identified as *M. neogunnii*, containing many bioactive compounds, could be used as the medicinal potential in human healthcare.

Keywords: Molecular phylogeny; morphological identification; entomopathogenic fungi; adenosine; cordycepin.

INTRODUCTION

The genus *Cordyceps* sensu lato (*Cordyceps* s.l.), which consists of more than 600 entomopathogenic fungi, have been used as herbal medicines for a long time (Kuo *et al*., 2015; Li *et al*., 2020). Recently, based on the phylogenetic analysis, *Cordyceps* s.l. was divived again into four genera, including *Cordyceps sensu stricto* (*Cordyceps* s.s, belonged to the family of *Cordycipitaceae*), *Metacordyceps* (belonged to the family *Clavicipitaceae*), *Ophiocordyceps* (belonged to the family *Ophiocordycipitacea*) and *Elaphocordyceps* (belonged to the family *Ophiocordycipitaceae*) (Sung *et al*., 2007). Of

thesespecies, *Metacordyceps neogunnii* T.C. Wen & K.D. Hyde belongs to the genus *Metacordyceps*. The genus *Metacordyceps* includes only a limit known species, remaining one of the most poorly understood. *Metacordyceps neogunnii* was reported to differ from others related *Metacordyceps* species mainly in having longer asci and wider ascospores (Wen *et al*., 2017). *Metacordyceps neogunnii* has been wrongly recognized as herbal entomopathogenic fungi *Cordyceps gunii* (Berk.) Berk. in China for more than 30 years (Wen *et al*., 2017; She *et al*., 2019). In the study of Wen *et al*. (2017), the fungus named "*Cordyceps gunnii*" in China has been correctly classified as *Metacordyceps neogunnii* based on the morphology analysis and combined multi-gene phylogenetic analysis (Wen *et al*., 2017). *Cordyceps gunnii* (Berk.) Berk. is known only from Australia (Berkeley, 1848). "*Cordyceps gunnii*" is morphologically and combined multigene phylogentically different to *Cordyceps gunnii* of Tasmania (an island state of Australia). *Cordyceps gunnii* of Tasmania also shows them to differ and different genus belonged to the family *Ophiocordycipitaceae* (Wen *et al*., 2017). "*Cordyceps gunnii*" has been reported to have chemical position and medical value similar to those of traditional *Cordyceps sinensis* in China (Zhu *et al*., 2013, 2016). Additionally, it has various medical effects, such as anti-tumor, anti-aging, promoting sleep and enhancing memory (Meng*et al*., 2019; She *et al*., 2019; Zhu *et al*., 2013, 2016). Therefore, "*Cordyceps gunnii*" has been used as a medicinal mushroom by local people in China (Zhu *et al*., 2013, 2016; She *et al*., 2019). Recently, several important secondary metabolites have been found in "*Cordyceps gunnii*", including polysaccharide, isoflavone, cordycepin, adenosine, anti- ultraviolet radiation components (Kuo *et al*., 2015; Zhu *et al*., 2016; She *et al*., 2019). These secondary metabolites have been shown to have pharmacological potential, and could be used as herbal medicines to enhance human health (She *et al*., 2019). For this reason, the search for entomopathogenic fungi diversity, including "*Cordyceps gunnii*", may provide an insight into the preventive and therapeutic potentials of these fungi for the biotechnological research as well as development of potential product. Vietnam is located in a tropical region with terrestrial ecosystems. The forests feature a rich biodiversity of both flora and fauna due to the tropical monsoon climate with high temperature and rainfall. This is a favorable environment for the development of entomopathogenic fungi. Lang Biang Biosphere Reserve is located in Lam Dong Province and comprises a vast primitive jungle with the Lang Biang Mountain at its core, one of Vietnam's four biodiversity centers. During our expedition to discover the diversity of entomopathogenic fungi, we have collected the sample DL0091.

In this study, species DL0091 was morphologically and phylogenetically described as *Metacordyceps neogunnii*, containing numerous bioactive constituents, especially a high amount of adenosine and cordycepin, therefore, it was considered a valuable resource in medicine.

MATERIALS AND METHODS

Fungal sample collection

The specimen, DL0091, used for this study was collected from Lang Biang Biosphere Reserve (elevation 1640 – 1750m) from May to October 2018. The specimen, including the host, was extracted carefully, noted, and photographed in the field using a digital camera. The specimen was immediately wrapped in wax paper, placed in a collection bag, and taken to the laboratory.

Morphology analysis

Morphological observations were carried out and recorded according to the guidelines of Kobayashi (1941; 1982) and Sung *et al*. (2007) (Kobayashi, 1941, 1982; Sung *et al*., 2007). The macroscopic characteristics of the fresh fruit body were carefully observed, including the stipe, stroma, etc. Additionally, the host insect was identified based on morphological characteristics, such as mandibulate mouth parts, antennae, shape of head and thorax. For the

micromorphological analysis, one or two perithecia were removed from the stroma and placed on a microscope slide in lactophenol-cotton blue to measure the sizes and shapes of the perithecia, asci and ascospores.

DNA extraction, PCR amplification, target gene sequencing

Genomic DNA was isolated by using the phenol/chloroform method (pH = 8) (Chomczynski, 1993). The fruiting body was incubated in a lysis buffer (2.0% SDS, Tris-HCl pH 8.0, 150 mM NaCl, 10mM EDTA, 0.1 mg/mL Proteinase K) at 65ºC overnight. The supernatant was collected by centrifugation, and a volume of 700 µL of phenol/chloroform/isoamyl alcohol (25:24:1) was supplemented and centrifuged. The supernatant was collected and precipitated with absolute isopropanol. Finally, the isolated genomic DNA was stored in Tris-EDTA buffer at –20ºC for further studies.

The primer pairs used to amplify *ITS*, *Tef*, *rpb1* gene were shown in Table 1. The final volume of PCR was done in a total of 15 µL with the thermal program: 1 cycle at 95ºC for 5 min, 40 cycles at 95ºC for 30 s, 55ºC for 30 s, 72ºC for 2 min, 1 cycle at 72ºC for 5 min. Five µL aliquots of amplification product were electrophoresed on a 2.0% agarose gel and visualized in a UV trans illuminator. The amplified product was sequenced by Sanger method.

Table 1. The primers' sequences used in current study.

Target gene	Primer	Sequence (5'-3')
ITS	ITS1F (F)	CTTGGTCATTTAGAGGAAGTAA
	ITS4 (R)	TCCTCCGCTTATTGATATGC
Tef	983F (F)	GCYCCYGGHCAYCGTGAYTTYAT
	2218R (R)	ATGACACCRACRGCRACRGTYTG
Rpb1	CRPB1 (F)	CCWGGYTTYATCAAGAARGT
	RPB1Cr (R)	CCNGCDATNTCRTTRTCCATRTA

Taxa and *ITS*, *Tef*, *Rpb1* sequences collection, and phylogenetic analysis

The data set of *ITS*, *Tef* and *Rpb1* sequences were established by sequences downloaded from Genbank (NCBI) and based on the previous data published by Sung *et al*. (2007) and Wen *et al*. (2017). The *ITS*, *Tef* and *Rpb1* were noted with accession number and name of taxon. The amplified DNA sequences were proofread to remove ambiguous signals at both ends by different software, including Seaview 4.2.12 and Chromas Lite 2.1.1. The phylogenetic tree was constructed based on neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and UPGMA (UP) using Molecular Evolutionary Genetics Analysis (MEGA) version 5.

Chemical constituents and bioactive compound analysis

For determination of chemical positions of specimen DL0091, powder of specimen DL0091 was sent for analysis at Center of Analytical Services and Experimentation HCMC, Vietnam (www.case.com.vn).

RESULTS

Taxonomy

Metacordyceps neogunnii (Figs. 1, 2)

Typification: VIETNAM. Lam Dong Province, Lang Biang Biosphere Reserve, Lang Biang mountain. Elevation 1640 – 1750 m; humidity: over 85%; temperature: day 20ºC – 22ºC, night: 14ºC – 16ºC; collected in May – October 2018, from the

larvae of *Lepidoptera* in moist soil surrounded by dried leaves (Figs. 1A and B).

Host: On the larva of *Lepidoptera*, 40 – 60 mm × 4 – 7 mm, buried in the soil (Figs. 1C and 2A).

Habitat: Individuals of associated species appeared at the type locality, including pioneer species such as *Acer laurinum* (*Aceraceae*), *Baccaurea harmandii* (*Euphorbiaceae*), *Castanopsis chinensis* (*Fagaceae*), *Eriobotrya poilanei* (*Rosaceae*), *Jasminum longisepalum* (*Oleaceae*), *Phoebe petelotii* (*Lauraceae*) and *Tetrastigma lanceolarium* (*Vitaceae*).

Stromata: arose from head of host, fleshy, rather tough, rarely branched (solitary or in group of two stromata), white to grey (Fig. 1), 15 – 130 mm × 2 – 6 mm (Figs. 1C and 2A, E). *Stipe*: cylindrical, 15 – 100 mm × 2 – 4 mm, white (the part in underground) to grey (the above part), fleshy, enlarging abruptly at fertile part (Figs. 1C and 2A). *Fertile part*: cylindrical or obtuse, round head shape, white (in young) to grey (in mature), 10 – 30 mm × 4 – 6 mm (Figs. 2A and B); Surface: grey with several irregular striate, black dots (Fig. 2C); cortex: white (Fig. 2D). *Perithecia*: immersed, elongated or ampuliform, even distribution, dark grey at the ostiole, 700 – 800 µm × 250 – 270 µm (Figs. 2F and G). *Asci*: cylindrical, hyaline, thick apical cap, 550 – 680 µm × 5 – 8 µm (Fig. 2H).

Ascospores: 3.0 – 4.0 µm × 1.8 – 2.1 µm, hyaline, filiform, multi-septate, disarticulating into secondary ascospores after released from the asci (Fig. 2I).

Phylogenetic analysis

The dataset of taxa in current study assembled from previously published studies (Sung *et al*., 2007; Wen *et al*., 2017), and were downloaded from GenBank (NCBI) for the construction of phylogenetic tree. We obtained 25 sequences of each *ITS*, *Tef* and *Rpb1* gene from 18 different species (Table 2). The combined dataset if three gene, *ITS*, *Tef* and *Rpb1* gene, consisted of 1127 bp and 24 taxa were analyzed, representing the genus *Metacordyceps* (*Clavicipitaceae*), *Cordyceps*, *Ophiocordyceps* and *Tolypocladium* (*Ophiocordycipitaceae*), the outgroup taxon *Glomerella cingulata* (*Glomerellaceae*, *Glomerellales*).

Figure 1. The DL0091 sample "*Metacordyceps neogunnii".* **A:** Stroma appeared in moist soil surrounded by dried leaves; **B:** Immature stroma; **C:** Variation size of stroma. Stromata arose from head of host.

Table 2. List of species and taxa used in this phylogenetic analysis.

Species	Vouchers	Accession number		
		ITS	***Tef***	***Rpb1***
Metacordyceps chlamydosporia	CBS 101244	JN049821	DQ522327	DQ522372
M. chlamydosporia	CBS 504.66	AJ292398	EF469069	EF469098
M. indigotica	TNS-F18553	JN049874	JF416010	JN049886
M. indigotica	TNS-F18554	JN049875	JF416011	JN049887
M. kusanagiensis	TNS F18494	JN049873	JF416014	JN049890
M. martialis	HMAS 197472(S)	JN049881	JF416016	JN049892
M. shibinensis	GZUH SB13050311	KR153585	KR153589	KR153590
M. taii	ARSEF 5714	JN049829	AF543775	DQ522383
M. yongmunensis	EFCC 2131	JN049856	EF468770	EF468876
M. neogunnii	BUM415	MH143811	MH143861	MH143876
M. neogunnii	GZUH SB13050301	KU729715	KU729726	KU729731
M. neogunnii	GZUH SB13050302	KU729716	KU729727	KU729732
M. neogunnii	GZUH SB13050304	KU729717	KU729728	KU729733
Cordyceps gunnii	Cs1	HM149352	HM149362	HM149367
C. gunnii	OSC 76404	JN049822	AY489616	AY489650
C. gunnii	ARSEF 6828	HM140630	HM140636	HM140639
Ophiocordyceps sinensis	EFCC 7287	JN049854	EF468767	EF468874
O. sinensis	ARSEF 6282	HM595981	HM595918	HM595952
O. stylophora	OSC 111000	JN049828	DQ522337	DQ522382
O. rhizoidea	N.H.J. 12522	JN049857	EF468764	EF468873
Tolypocladium japonica	OSC110991	JN049824	DQ522330	DQ522375
T. ophioglossoides	OSC106405	JN943320	AY489618	AY489652
T. subsessilis	OSC71235	JN049844	EF469061	EF469090
Glomerella cingulate	CBS 114054	DQ286202	AF543773	AY489659

In the phylogenetic analysis, the best model was TN93+G, -lnL = 6473.37, G=0.25. The parameters used included base frequencies – freqA = 0.24, freqT = 0.20, freqC = 0.30, and freqG = 0.26. The NJ, MP, ML and UP analyses showed the similar topologies resolving the taxonomic relationship between species DL0091 and others. The NJ, MP, ML and UP phylogenetic trees could be broadly separated into different genera: *Metacordyceps*, *Cordyceps*, *Tolypocladium*, and *Ophiocordyceps* (Fig. 3).

The species DL0091 and taxon *Cordyceps gunnii*from China (*ITS*: HM149352, *Tef*: HM149362, and *Rpb1*: HM149367) the separate *Metacordyceps neogunnii* clade with other species of *Metacordyceps neogunnii* with credible bootstrap support (NJ: 96, MP: 100, ML: 100, UPMA: 100) (Fig. 4). The clade of *Metacordyceps neogunnii* from the well-supported separate clade of *Metacordyceps* genus with other species of *Metacordyceps* in the family of *Clavicipitaceae*, including *Metacordyceps chlamydosporia*, *Metacordyceps indigotica*, *Metacordyceps kusanagiensis*, *Metacordyceps martialis*, *Metacordyceps shibinensis*, *Metacordyceps*

taii, *Metacordyceps yongmunensis* (NJ: 99, MP: 100, ML: 100, UPMA: 99) (Fig. 4). Two specimens of *Cordyceps gunnii* from Tasmania (Australia) formed a separate clade of *Cordyceps* genus with well-supported value (NJ: 100, MP: 100, ML: 100, UPMA: 100), closely to the genus of *Metacordyceps* and *Tolipocladium* (Fig. 4).

Figure 2. Morphology analysis of DL0091 "*Metacordyceps neogunnii*". **A:** Stroma; **B:** Fertile part; **C:** Surface of fertile part; **D:** White cortex; **E:** Host: larva of *Lepidoptera*; **F, G:** Perithecia; **H:** Asci, thick apical cap; **I:** Ascospores.

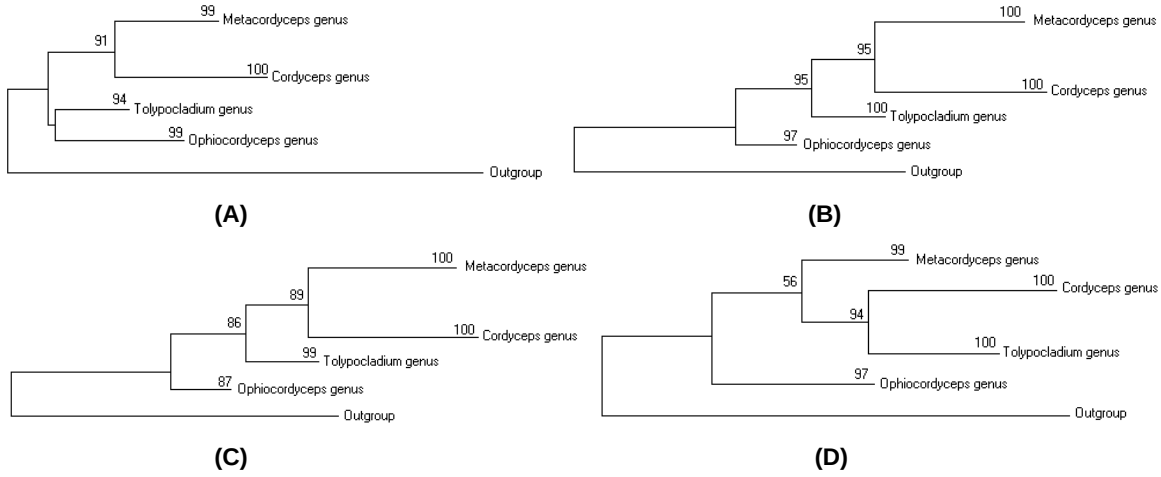
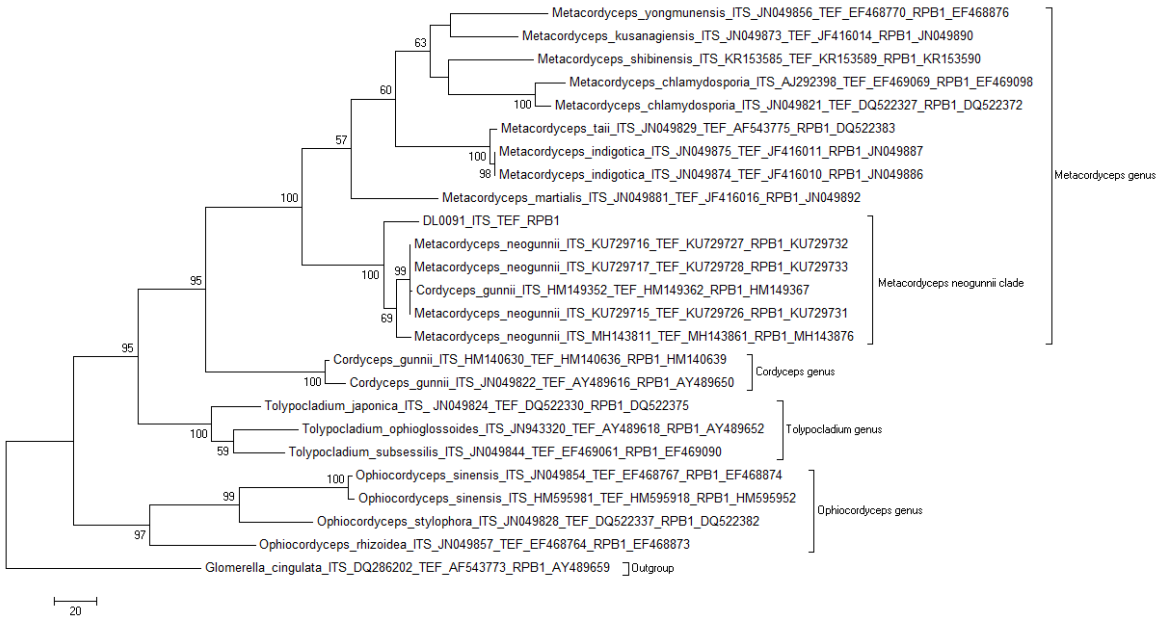


Figure 3. Schematic diagrams of phylogenetic relationships. **A:** Neighbor joining; **B:** Maximum parsimony; **C:** Maximum likelihood (ML), UPGMA in genus sampling. Bootstrap: 1000 replicates. The bootstrap value was indicated above nodes. The tree is rooted to *Glomerella cingulate* (Outgroup).


Metacordyceps_yongmunensis_ITS_JN049856_TEF_EF468770_RPB1_EF468876
Metacordyceps_kusanagiensis_ITS_JN049873_TEF_JF416014_RPB1_JN049890
Metacordyceps_shibinensis_ITS_KR153585_TEF_KR153589_RPB1_KR153590
Metacordyceps_chlamydosporia_ITS_AJ292398_TEF_EF469069_RPB1_EF469098
Metacordyceps_chlamydosporia_ITS_JN049821_TEF_DQ522327_RPB1_DQ522372
Metacordyceps_taii_ITS_JN049829_TEF_AF543775_RPB1_DQ522383
Metacordyceps_indigotica_ITS_JN049875_TEF_JF416011_RPB1_JN049887
Metacordyceps_indigotica_ITS_JN049874_TEF_JF416010_RPB1_JN049886
Metacordyceps_martialis_ITS_JN049881_TEF_JF416016_RPB1_JN049892
DL0091_ITS_TEF_RPB1
Metacordyceps_neogunnii_ITS_KU729716_TEF_KU729727_RPB1_KU729732
Metacordyceps_neogunnii_ITS_KU729717_TEF_KU729728_RPB1_KU729733
Cordyceps_gunnii_ITS_HM149352_TEF_HM149362_RPB1_HM149367
Metacordyceps_neogunnii_ITS_KU729715_TEF_KU729726_RPB1_KU729731
Metacordyceps_neogunnii_ITS_MH143811_TEF_MH143861_RPB1_MH143876
Cordyceps_gunnii_ITS_HM140630_TEF_HM140636_RPB1_HM140639
Cordyceps_gunnii_ITS_JN049822_TEF_AY489616_RPB1_AY489650
Tolypocladium_japonica_ITS_ JN049824_TEF_DQ522330_RPB1_DQ522375
Tolypocladium_ophioglossoides_ITS_JN943320_TEF_AY489618_RPB1_AY489652
Tolypocladium_subsessilis_ITS_JN049844_TEF_EF469061_RPB1_EF469090
Ophiocordyceps_sinensis_ITS_JN049854_TEF_EF468767_RPB1_EF468874
Ophiocordyceps_sinensis_ITS_HM595981_TEF_HM595918_RPB1_HM595952
Ophiocordyceps_stylophora_ITS_JN049828_TEF_DQ522337_RPB1_DQ522382
Ophiocordyceps_rhizoidea_ITS_JN049857_TEF_EF468764_RPB1_EF468873
Glomerella_cingulata_ITS_DQ286202_TEF_AF543773_RPB1_AY489659
Metacordyceps genus
Metacordyceps neogunnii clade
Cordyceps genus
Tolypocladium genus
Ophiocordyceps genus
Outgroup
20

(A)


Metacordyceps_yongmunensis_ITS_JN049856_TEF_EF468770_RPB1_EF468876
Metacordyceps_chlamydosporia_ITS_AJ292398_TEF_EF469069_RPB1_EF469098
Metacordyceps_chlamydosporia_ITS_JN049821_TEF_DQ522327_RPB1_DQ522372
Metacordyceps_shibinensis_ITS_KR153585_TEF_KR153589_RPB1_KR153590
Metacordyceps_kusanagiensis_ITS_JN049873_TEF_JF416014_RPB1_JN049890
Metacordyceps_taii_ITS_JN049829_TEF_AF543775_RPB1_DQ522383
Metacordyceps_indigotica_ITS_JN049875_TEF_JF416011_RPB1_JN049887
Metacordyceps_indigotica_ITS_JN049874_TEF_JF416010_RPB1_JN049886
Cordyceps_gunnii_ITS_HM149352_TEF_HM149362_RPB1_HM149367
Metacordyceps_neogunnii_ITS_KU729716_TEF_KU729727_RPB1_KU729732
Metacordyceps_neogunnii_ITS_KU729715_TEF_KU729726_RPB1_KU729731
Metacordyceps_neogunnii_ITS_KU729717_TEF_KU729728_RPB1_KU729733
DL0091_ITS_TEF_RPB1
Metacordyceps_neogunnii_ITS_MH143811_TEF_MH143861_RPB1_MH143876
Metacordyceps_martialis_ITS_JN049881_TEF_JF416016_RPB1_JN049892
Metacordyceps genus
Metacordyceps neogunnii clade

(B)


Metacordyceps_yongmunensis_ITS_JN049856_TEF_EF468770_RPB1_EF468876
Metacordyceps_kusanagiensis_ITS_JN049873_TEF_JF416014_RPB1_JN049890
Metacordyceps_shibinensis_ITS_KR153585_TEF_KR153589_RPB1_KR153590
Metacordyceps_chlamydosporia_ITS_AJ292398_TEF_EF469069_RPB1_EF469098
Metacordyceps_chlamydosporia_ITS_JN049821_TEF_DQ522327_RPB1_DQ522372
Metacordyceps_taii_ITS_JN049829_TEF_AF543775_RPB1_DQ522383
Metacordyceps_indigotica_ITS_JN049875_TEF_JF416011_RPB1_JN049887
Metacordyceps_indigotica_ITS_JN049874_TEF_JF416010_RPB1_JN049886
Metacordyceps_martialis_ITS_JN049881_TEF_JF416016_RPB1_JN049892
DL0091_ITS_TEF_RPB1
Metacordyceps_neogunnii_ITS_KU729716_TEF_KU729727_RPB1_KU729732
Metacordyceps_neogunnii_ITS_KU729717_TEF_KU729728_RPB1_KU729733
Cordyceps_gunnii_ITS_HM149352_TEF_HM149362_RPB1_HM149367
Metacordyceps_neogunnii_ITS_KU729715_TEF_KU729726_RPB1_KU729731
Metacordyceps_neogunnii_ITS_MH143811_TEF_MH143861_RPB1_MH143876
Metacordyceps genus
Metacordyceps neogunnii clade

(C)

(D)

Figure 4. Schematic diagram of phylogenetic analysis. **A:** Phylogenetic relationships among DL0091 and related species based on combined analysis of *ITS*, *Tef*, *Rpb1* data from ML analysis; **B:** Enlargement of *Metacordyceps* genus of Neighbor joining; **C:** Maximum likelihood; **D:** UPGMA tree. Bootstrap: 1000 replicates. The bootstrap value was indicated above nodes. The tree is rooted to *Glomerella cingulate* (Outgroup).

Chemical and bioactive compounds analysis

Numerous bioactive constituents, such as cordycepin, adenosine, polysaccharides, phytosterol, as well as other chemical positions, such as protein, amino acid, ash, fat, carbohydrate, have been extracted from specimen DL0091. The bioactive constituents and other chemical positions were shown in Table 4.

Table 3. Synopsis of the characteristics of DL0091 and related species.

Species	Host	Stromata	Ascomata	Asci	Ascospores	Reference
DL0091	Larvae of *Lepidoptera*	Fleshy, white to grey, rarely branched, 15–130 mm x 2-6 mm	Embeded	Cylindrical, hyaline, 550–680 µm x 5–8 µm, thick apical cap	3.0–4.0 µm x 1.8–2.1 µm, hyaline, filiform, multi-septate, disarticulating into secondary ascospores	This study
*Metacordycepsneogunnii*T.C. Wen & K.D. Hyde	Larvae of *Lepidoptera*	Fleshy, white to gray, rarely branched, 40–80 mm × 2-6 mm	Embedded	Cylindrical, hyaline, 250-480 × 3–5, possessing a prominent apical cap	330-460 µm × 2–3 µm, hyaline, filiform, multi-septate, breaking into secondary ascospores	Wen, T.C *et al* (2017)
Cordyceps gunnii (Berk.) Berk	Larvae of *Endoclita excrescens*	Singularia, calvata, ecaptehospite; Stipe 36.6–52.3 mm × 4.8–8.6 mm, head 18.5–19.3 mm × 4–9.4 mm	Embedded	Cylindrical, 345–530 × 4.4–6.9	Hyaline, filiform, multi-septate, breaking into 2–4.3 µm × 1–1.8 µm secondary ascospores	Li, Z. *et al* (1999)

Table 4. Chemical positions of DL0091.

No.	Parameters	Unit	Amount
1	Fat	%	3.75
2	Protein	%	27.6
3	Ash	%	5.06
4	Carbohydrate	%	2.51
5	Polysaccharide	%	3.71
6	Amino acid	g/100 g	13.30
	Alanine	g/100 g	1.14
	Arginine	g/100 g	0.94
	Aspartic acid	g/100 g	1.54
	Glutamic acid	g/100 g	1.88
	Glycine	g/100 g	0.71
	Histidine	g/100 g	0.34
	Isoleucine	g/100 g	0.54
	Leucine	g/100 g	0.83
	Lysine	g/100 g	1.23
	Methionine	g/100 g	0.12
	Phenylalanine	g/100 g	0.47
	Proline	g/100 g	0.64
	Serine	g/100 g	0.74
	Threonine	g/100 g	0.84
	Tyrosine	g/100 g	0.44
	Valine	g/100 g	0.91
7	Phytosterol		
	Campesterol	mg/100 g	474
	Beta-sistosterol	mg/100 g	13.5
8	Adenosine	mg/Kg	634
9	Cordycepin	mg/Kg	35.2

DISCUSSION

In the field of pharmaceutical industry and traditional medicine fields, the search for the natural resources, especially entomopathogenic fungi, has been considered to be important to develop biological product. Lang Biang Biosphere Reserve, located in Lam Dong Province, is classified as Vietnam's biodiversity center and considered a hotspot of fungal biodiversity, including entomopathogenic fungi. During our expedition to validate the diversity of entomopathogenic fungi in Lang Biang Biosphere Reserve, the species DL0091 was collected. Morphologically, the species DL0091 was identified as *Metacordyceps neogunnii*, belonged to *Metacordyceps* genus, *Clavicipitaceae* family. The species DL0091 shared the common characteristics of the genus of *Metacordyceps*. The genus of *Metacordyceps* G.H. Sung, et al. was first introduced by Sung *et al* (2007) which was characterized by (1) stromata: solitary, simple or branched; (2) stipe: fleshly or touch, cylindrical to enlarging in fertile

part; (3) perithecia: partially or completely immersed in stromata, ordinal or oblique in arrangement; (4) asci: cylindrical, thickened ascus apex; (5) ascospores: cylindrical, multiseptate, disarticulating into part-spores; (6) host: almost always buried in soil (Sung *et al*., 2007). Compared to species belonged to the genus *Metacordyceps*, DL0091 was similar to *Metacordyceps neogunnii* T.C. Wen & K.D. Hyde, and different from the species *Cordyceps gunnii* (Berk.) Berk (Table 3). Here, the phylogenetic trees were conducted based on the combined sequence data from multi-gene loci, including *ITS*, *Tef* and *Rpb1*. Phylogenetically, the species DL0091 clustered with *Metacordyceps neogunnii*, and *Cordyceps gunnii* from China (*ITS*: HM149352, *Tef*: HM149362, and *Rpb1*: HM149367) to form the separate *Metacordyceps neogunnii* clade (belonged to the genus *Metacordyceps*) with well-supported bootstrap samplings. The taxon *Cordyceps gunnii* from China was clustered within this group, it could be explained that it was wrongly classified as *Cordyceps gunnii* for more than 30 years, and has been correctly classified as *Metacordyceps neogunnii* based on the morphology analysis and combined multi-gene phylogenetic analysis (Wen *et al*., 2017). The correct *Cordyceps gunnii* from Tasmania (Australia) formed a clade that split a separate clade from *Metacordyceps neogunnii* clade with well-supported nodal value.

This study conclusively demonstrates that species DL0091, collected from Lang Biang Biosphere Reserve, Vietnam, was *Metacordyceps neogunnii* based on the morphology and phylogenetic analysis. Additionally, this is the first record of *Metacordyceps neogunnii* in Vietnam. Due to the bioactivities of *Metacordyceps neogunnii*, the record of DL0091 as *Metacordyceps neogunnii* may provide an insight into the preventive and therapeutic potentials of this fungi for the biotechnological research as well as development of potential product. Moreover, the strategies used for identifying *Cordyceps* species based on combined morphology analysis and phylogenetic analysis based on multi-gene loci could have a wide application in entomopathogenic fungi.

Generally, many chemical constituents of specimen DL0091 have been identified including protein, fat, carbohydrate, amino acid, cordycepin and adenosine, therefore, the understandings of chemical compositions in this study could apply in the development of new drugs and therapeutics. Amount of protein, fat, ash, carbohydrate, polysaccharide were 27.6%, 3.75%, 5.06%, 2.51% and 3.71. Essential and non-essential amino acid shown in Table 4 revealed the presence of 16 amino acids with the amount of 13.30 f/100 g. Nine essential amino acids were detected, including Arginine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Valine, of which Arginine and Valine were the highest (0.94 g/100 g and 0.91 g/100 g, respectively). Seven non-essential amino acids were Alanine, Aspartic acid, Glutamic acid, Glycin, Proline, Serine, and Tyrosine, with the highest amount for Glutamic acid (1.88 g/100 g). Free phytosterol, including Campesterol and Beta-sistosterol, were identified in DL0091. It has been evidenced that Beta-sistosterol, a major phytosterol, possessed many biological activities, such as anti-tumorigenesis, anti-inflammatory, hepato-protective, antioxidant as well as anti-diabetic functions (Yang *et al*., 2009). Cordycepin and Adenosine concentration in DL0091 were presented in the amount of 634 mg/Kg, and 35.2 mg/Kg. Cordycepin and Adenosine were the categories of compounds that exhibited significant therapeutic potential, such as anti-inflammatory, analgesic, and regulation of immune response, anti-tumorigenesis, anti-metastatic, and anti-proliferative effects, as well as inducing apoptosis (Shin *et al*., 2009; Liu *et al*., 2015; Jin *et al*., 2018). Therefore, the nutritional and bioactive values of DL0091 detected indicated its potential use in medical application as well as source of development of functional food for healthcare.

CONCLUSION

We successfully applied morphological characterization in combination with phylogenetic analysis of multiple genes, including *ITS*, *Tef* and *Rpb1*, to delimit sample DL0091, collected from Lang Biang Biosphere Reserve located in Lam Dong Province, Vietnam, as *Metacordyceps neogunnii* (Wen *et al.*, 2017) belonging to genus *Metacordyceps*, family Clavicipitaceae. The first record of DL0091 as *Metacordyceps neogunnii* may provide an insight into the preventive and therapeutic potentials of this fungi for the biotechnological research as well as development of potential product. The detectable chemical constituents and bioactive values of DL0091 could be applied development of medical as well as source of development of functional food for healthcare.

Acknowledgments: *We express our special thanks to National Foundation for Science and Technology Development (NAFOSTED): under the grant number 106-NN.06.2015.44, Vietnam. We also thank Vo Thanh Nhan, Nguyen Thanh Tung for their assistance.*

REFERENCES

Berkeley MJ (1848) Decades of fungi. Decade XX. Tasmanian fungi. *London J Bot* 7: 572–580.

Chomczynski P (1993) Areagentforthesingle-stepsimultaneous *Biotechniques 15*(3): 536–537.

Jin Y, Meng X, Qiu Z, Su Y, Yu P, Qu P (2018) Anti-tumor and antimetastatic roles of cordycepin, one bioactive compound of *Cordyceps militaris*. *Saudi J Biol Sci* 25(5): 991-995.

Kobayashi Y (1941) The genus *Cordyceps* and its allies. *Sci Rep Tokyo Bunrika Daigaku Sect B*5: 53–260.

Kobayashi Y (1982) Keys to the taxa of the genera *Cordyceps* and *Torrubiella*. *Trans Mycol Soc Japan* 23: 329–364.

Kuo HC, Huang IC, Chen TY (2015) *Cordyceps* s.l. (ascomycetes) species used as medicinal mushrooms are closely related with higher ability to produce cordycepin. *Int J Med Mushrooms* 17(11): 1077–1085.

Li Y, Chen W, Han Y, Liang J, Liang Z (2020) *Cordyceps yinjiangensis, a newant-pathogenic fungus*. *Phytotaxa* 453(3): 284–292.

Li Z, Li C, Huang B, Fan M, Lee M (1999) new variety of *Cordyceps gunnii* (Berk.) Berk. and its paecilomyces anamorph. *Korean J Mycol* 27(3): 231-233.

Liu Y, Wang J, Wang W, Zhang H, Zhang X, Han C (2015) The chemicalconstituents and pharmacological actions of *Cordyceps sinensis. J Evid Compl Alter Med* 1–12.

Meng M, Wang H, Li Z, Guo M, Hou L (2019) Protective effects ofpolysaccharides from *Cordyceps gunnii* mycelia against cyclophosphamide-induced immunosuppression to TLR4/TRAF6/NF-κB signalling in BALB/c mice. *Food Funct* 10(6): 3262–3271.

She X, Yao T, Ding L, Gan Z, Tang M, Han Y (2019) Identification andanalysis of secondary metabolites from CH_3OH/H_2O extract of *Metacordyceps neogunnii*. *E3S Web Conf, 118*.

Shin S, Moon S, Park Y, Kwon J, Lee S, Lee CK, Cho K, Ha NJ, Kim K (2009) Role of cordycepin and adenosine on the phenotypic switch of macrophages via induced anti-inflammatory cytokines. *Immune Netw* 9(6): 255.

Sung GH, Hywel-Jones NL, Sung JM, Luangsaard JJ, Shrestha B, Spatafora JW (2007) Phylogenetic classification of *Cordyceps* and theclavicipitaceous fungi. *Stud Mycol* 57: 5–59.

Wen TC, Xiao YP, Han YF, HuangSK, Zha LS, Hyde KD, Kang JC (2017) Multigene phylogeny and morphology reveal that the Chinese medicinal mushroom '*Cordyceps gunnii'* is *Metacordyceps neogunnii* sp. Nov. *Phytotaxa* 302(1): 27–39.

Yang FQ, Feng K, Zhao J, Li SP (2009) Analysis of sterols and fatty acids innatural and cultured *Cordyceps* by one-step derivatization followed with gaschromatography–mass spectrometry. *J Pharmaceut Biomed* 49(5):1172–1178.

Zhu ZY, Liu XC, Tang YL, Dong FY, Sun HQ, Chen L, Zhang, YM (2016) Effects of cultural medium on the formation and antitumor activity ofpolysaccharides by *Cordyceps gunnii*. *J Biosci Bioeng* 122(4): 494–498.

Zhu ZY, Liu Y, Si CL, Yuan J, Lv Q, Li YY, DongGL, Liu AJ, Zhang YM (2013) Sulfated modification of the polysaccharide from *Cordyceps gunnii* mycelia and its biological activities. *Carbohydr Polym* 92(1): 872–876.