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

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SUMMARY

An entomopathogenic fungus, specimen DL0091 parasitized on the larva of Lepidoptera, was collected from Lang Biang Biosphere Reserve, located in Lam Dong Province, Vietnam. The specimen DL0091 has been analyzed to contain 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 divided 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 Ophiocordycipitaceae) and Elaphocordyceps (belonged to the family Ophiocordycipitaceae) (Sung et al., 2007).
The fungus named *Cordyceps gunnii*, including *Cordyceps neogunnii*, has been wrongly recognized as herbal entomopathogenic fungi *Cordyceps sinensis* (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 multigene phylogenetic analysis (Wen et al., 2017). *Cordyceps gunnii* (Berk.) Berk. is known only from Australia (Berkeley, 1848). “*Cordyceps gunnii*” is morphologically and combined multigene phylogenetically 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 (Menget 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 65°C 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 −20°C 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 95°C for 5 min, 40 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 2 min, 1 cycle at 72°C 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) | ATGACACCRCRACRGCRCRGTYTG |
| Rpb1        | CRPB1 (F) | CCWGGYTTYAATCAAGAARGT |
|             | RPB1Cr (R) | CCNGCDATNTCTTTRCCATRTA |

**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 20°C – 22°C, night: 14°C – 16°C; 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).

**Asciorspores:** 3.0 – 4.0 μm × 1.8 – 2.1 μm, hyaline, filiform, multi-septate, disarticulating into secondary ascospores after released from the asc (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* (*Glomerellales*).
In the phylogenetic analysis, the best model was TN93+G, \( -\ln L = 6473.37, G=0.25 \). The parameters used included base frequencies \( \text{freqA} = 0.24, \text{freqT} = 0.20, \text{freqC} = 0.30, \) and \( \text{freqG} = 0.26 \). 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 kusaniensis*, *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).
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                  | Ascospores                           | Reference                      |
|--------------------------------|-------------------------------------------|---------------------------------|---------------------------|--------------------------------------|--------------------------------|
| DL0091                         | Larvae of Lepidoptera                     | Fleshy, white to grey, rarely branched, 15–130 mm x 2–6 mm | Embedded                  | 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                        |
| Metacordyceps neogunnii Wen & K.D. Hyde | Larvae of Lepidoptera                     | Fleshy, white to grey, rarely branched, 40–80 mm x 2–6 mm | Embedded                  | Cylindrical, hyaline, 250–480 x 3–5, possessing a prominent apical cap | 330–460 µm x 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            | Singulicaria, calvata, ecapite hospite; Stipe 36.6–52.3 mm x 4.8–8.6 mm, head 18.5–19.3 mm x 4–9.4 mm | Embedded                  | Cylindrical, 345–530 x 4.4–6.9 | Hyaline, filiform, multi-septate, breaking into 2–4.5 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, multisepitate, 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, hepatoprotective, 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 Rpbl, 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.

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