# The phylogenetic authentication of *Ophiocordyceps sphecocephala* from Lang Biang Biosphere Reserve, Lam Dong, Vietnam

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**ABSTRACT**

During our expedition at Lang Biang Biosphere Reserve, samples of DL0023, parasitizing on wasps, were collected, then sent to the biology laboratory, Dalat University, Lam Dong Province, Vietnam, to preliminarily morphologically identify the name of this species as *Ophiocordyceps sphecocephala*. The method of Phenol (pH = 8)/Chloroform method was applied to isolated DNA. The PCR assays and sequencing were performed to amplify and sequencing *ITS*, *nrLSU*, and *nrSSU*, respectively. The phylogenetic methods of neighbor-joining, maximum likelihood, and maximum parsimony were constructed to identify the relationship between DL0023 and referent sequences. We successfully amplified and sequenced the target genes, and constructed concatenated data containing 29, 33, and 30 sequences of *ITS*, *nrLSU* and *nrSSU*, respectively, belonging to *Clavicipitaceae* clade A, clade B and clade C, and 02 sequences belonged to *Glomerellaceae*. The results of phylogenetic analysis shown that DL0023 was accepted at Ophiocordyceps and strongly formed the monophyletic group *Ophiocordyceps sphecocephala* with a high supported value. In conclusion, the concatenated dataset's phylogenetic analyses effectively supported the identification of DL0023 as *Ophiocordyceps sphecocephala*.

## 1. Introduction

The genus of *Ophiocordyceps* (Hypocreales, Ascomycota) comprises fungal species parasitizing arthropods, which was originally described by Petch in 1913 (Tasanathai et al., 2019). Species of *Ophiocordyceps* were described as being present on a wide range of insect hosts, including *Lepidoptera*, *Neuroptera*, *Diptera*, *Hemiptera*, *Coleoptera*, *Hymenoptera*, *Isoptera*, and *Odonata* (Luangsa-Ard, Tasanathai, Thanakitpipattana, Khonsanit, & Stadler, 2018). *Ophiocordyceps* has a pan-global distribution. However, reports indicate that the tropics and subtropics have the largest species diversity (Luangsa-Ard et al., 2018). Furthermore, bioactive comp ounds have been found in various species of *Ophiocordyceps*, such as *Ophiocordyceps sinensis*, *Ophiocordyceps xuefengensis*, *Ophiocordyceps sobolifera*, etc. with a wide range of pharmacological activities (Jin et al., 2018; Le et al., 2022; Xu et al., 2016). Unsustainable and widespread harvesting of this caterpillar-fungus parasitic species has been reported to be causing threats to these species (Hopping, Chignell, & Lambin, 2018, Lao, Le, & Truong, 2021). Thus, research on species of *Ophiocordyceps* is vital to generating insightful data for conserving these fungi and monitoring biodiversity.

*Ophiocordyceps sphecocephala*, synonyms: *Sphaeria sphecocephala* Klotzsch ex Berk., J. Bot. (Hooker) 2: 206 (1843); *Cordyceps sphecophila* (Klotzsch ex Berk.) Berk. & M.A. Curtis, J. Linn., Soc. Bot. 10: 376. (1868), is an entomopathogenic fungi parasitizing on bees or wasps, belongs to the genus of *Ophiocordyceps*, the order of *Hypocreales*, and the family of *Clavicipitaceae* (Sung et al., 2007). *Ophiocordyceps sphecocephala* has been potential as a novel therapeutic agent for cancer treatment, anti-asthmatic activities, and so on (Heo et al., 2010; Oh et al., 2008). It is critical to creating procedures for authenticating *Ophiocordyceps sphecocephala* since species identification is a significant and vital process for controlling the quality and uniformity of herbal medicines. Notably, there is still a paucity of the molecular database applied for molecular-based authentication. Therefore, we investigated the molecular database as well as phylogenetic method for identifying *Ophiocordyceps sphecocephala*, using a combined analysis of three loci, including *ITS*, *nrLSU*, *nrSSU*.

## 2. Materials and methods

### *2.1. Sample collection*

We collected a sample of DL0023, a wasp parasite, at Lang Biang Biosphere Reserve (N12º2'19.0", E108º26'04.7", elevation 1680m) through the expedition aiming to evaluate the entomopathogenic fungi' diversity.

### *2.2. DNA extraction, PCR assay for target sequencing*

A total of DNA was isolated from dried samples according to the method of phenol/chloroform (pH = 8) with the addition of beta-mercaptoethanol. (Chomczynski & Sacchi, 1987). The material was mixed with a lysis solution consisting of Tris-HCl pH 8.0, 2.0% SDS, 150mM NaCl, 10mM EDTA, and 0.1 mg/mL Proteinase K. It was fully blended by inverting the tube many times over overnight incubation at 65°C. The upper phase was collected by centrifuging at 10,000 rpm/minute, for 10 minutes.

700µL Phenol/Chloroform/Isoamyl alcohol (ratio: 25:24:1) was added and centrifuged. After being centrifuged, the upper phase was precipitated with 99% ethanol, and DNA was precipitated by the Isopropanol method. The concentration of DNA was assessed by the method of $OD_{260}$. For further studies, isolated DNA was stored in Tris-EDTA at -20°C.

The primers amplifying the genes of *ITS*, *nrLSU*, and *nrSSU* are listed in Table 1. The protocol of PCR assay was performed according to the following cycle: 1 cycle at 95°C for 5 minutes, 40 cycles at 95°C for 30s, X°C for 30s, 72°C for 2 minutes, 1 cycle at 72°C for 5 minutes. 5µL aliquots of amplification product were electrophoresed on a 2.0% agarose gel and visualized. The amplified product was sequenced at Nam Khoa company.

**Table 1**

The primers used in the current study

| Targets | Primer | Sequence (5'-3') | XºC | References |
|---|---|---|---|---|
| *ITS* | ITS1 (F) | CTTGGTCATTTAGAGGAAGTAA | 55 | Gardes and Bruns (1993) |
| | ITS4 (R) | TCCTCCGCTTATTGATATGC | | |
| *nrLSU* | LR0R | GTACCCGCTGAACTTAAGC | 55 | Sung et al. (2007) |
| | LR5 | ATCCTGAGGGAAACTTC | | |
| *nrSSU* | NS1 | GTAGTCATATGCTTGTCTC | 42.5 | |
| | NS4 | CTTCCGTCAATTCCTTTAAG | | |

Note: X°C is the annealing temperatures for each target gene

### *2.3. Databases of ITS, nrLSU, and nrSSU, DNA proofreading and phylogeny analysis*

The databases of *ITS*, *nrLSU*, and *nrSSU* were collected from Genbank (NBCI) based on the previous data published by Sung et al. (2007). All sequences were collected with the following information: accession number, name of a taxon, and locality. The ambiguous signals of the amplified DNA sequences were removed or proofread by using Seaview 4.2.12 and Chromas Lite 2.1.1 and MEGA (Molecular Evolutionary Genetics Analysis). Finally, the phylogenetic trees were constructed under the phylogenetic methods: Neighbor-Joining (NJ), Maximum Parsimony (MP), and Maximum Likelihood (ML), according to the guideline of MEGA.

## 3. Results

### *3.1. Amplification of targets: ITS, nrLSU, and nrSSU*

The samples of DL0023 were preliminarily morphologically identified as *Ophiocordyceps sphecocephala*. Then, the genomic DNA was isolated, and target sequences were amplified according to the above description; then, electrophoresis on 2.0% agarose gel, which showed a significant and clear band of 600bps, 950bps, 1000bps for *ITS*, *nrLSU*, and *nrSSU*, respectively (Figure 1). The amplified product was sequenced, then, ambiguous signals were removed and proofread, as the result, sequencing signals from target genes' strands were distinct and good for reading (data not shown). According to BLAST results, the *ITS*, *nrLSU*, and *nrSSU* of DL0023 exhibited > 90% similarity to the *ITS*, *nrLSU*, and *nrSSU* of *Ophiocordyceps sphecocephala* (Accession number: AJ536550).

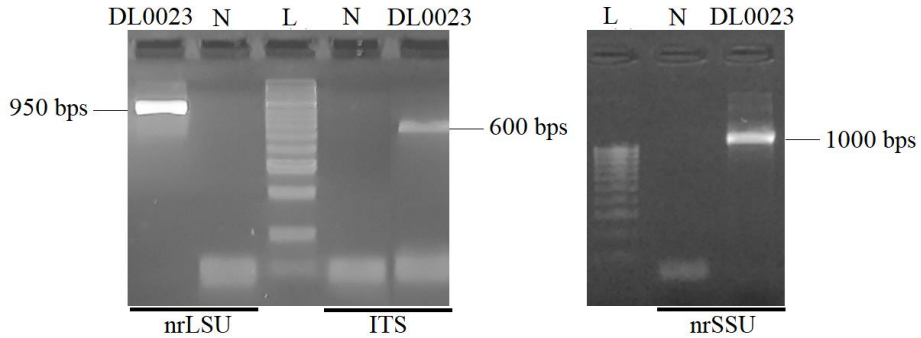



**Figure 1.** Electrophoresis of amplified sequences of DL0023

### *3.2. The database of target genes and phylogeny analysis*

A referent dataset of 29, 33, and 30 sequences of *ITS*, *nrLSU*, and *nrSSU*, respectively, belonging to *Clavicipitaceae*, *Cordicipitaceae*, and *Ophiocordycipitaceae*, and 02 outgroup sequences belonged to *Glomerellaceae* were collected from Genbank and sequences of DL0023 (Table 2). As the result, according to the database, they were divided into three clades: *Clavicipitaceae* clade A, clade B, and clade C within their separated subclades.

The model of best-fit evolution for *ITS*, *nrLSU*, *nrSSU*, as well as *ITS-nrLSU-nrSSU* were obtained by using the MEGA tool, shown in Table 2. The following models were applied to build the phylogenetic trees by the methods of Maximum likelihood, Neighbour joining, and Maximum pasimony from the concatenated data set. The family of *Clavicipitaceae* clade B formed the individual group and separated from others. According to the sample of DL0023, DL0023 formed the monophyletic group with referent taxon: *Ophiocordyceps sphecocephala* within the high supported bootstrap values (100 for individual genes analysis, as well as multiple-genes analysis by NJ/ML/MP), presented in Figure 2 and Figure 3.

**Table 2**

The best-fit evolution model of target genes' analysis

| Genes | Model, parameters |
|---|---|
| *ITS* | K2+G, BIC = 2336.960, AICc = 1980.897, InL = -934.813 |
| *nrLSU* | TN93+G, BIC = 8885.591, AICc = 8421.693, InL = -4151.663 |
| *nrSSU* | K2+G, BIC = 5340.193, AICc = 4914.001, InL = -2401.822 |
| *ITS-nrLSU-nrSSU* | TN93+G, BIC = 16431.483, AICc = 15925.994, InL = -7903.906 |

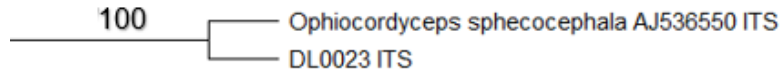
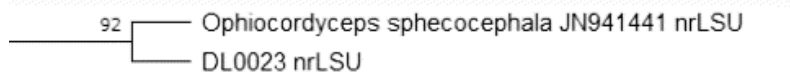
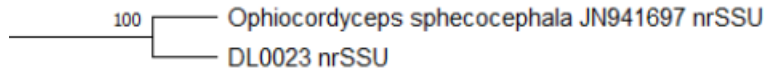
| Gene | Bootstrap value (MP) |
|---|---|
| *ITS* |   |
| *nrLSU* |   |
| *nrSSU* |   |

**Figure 2.** DL0023 clustered together with *Ophiocordyceps sphecocephala* with bootstrap support

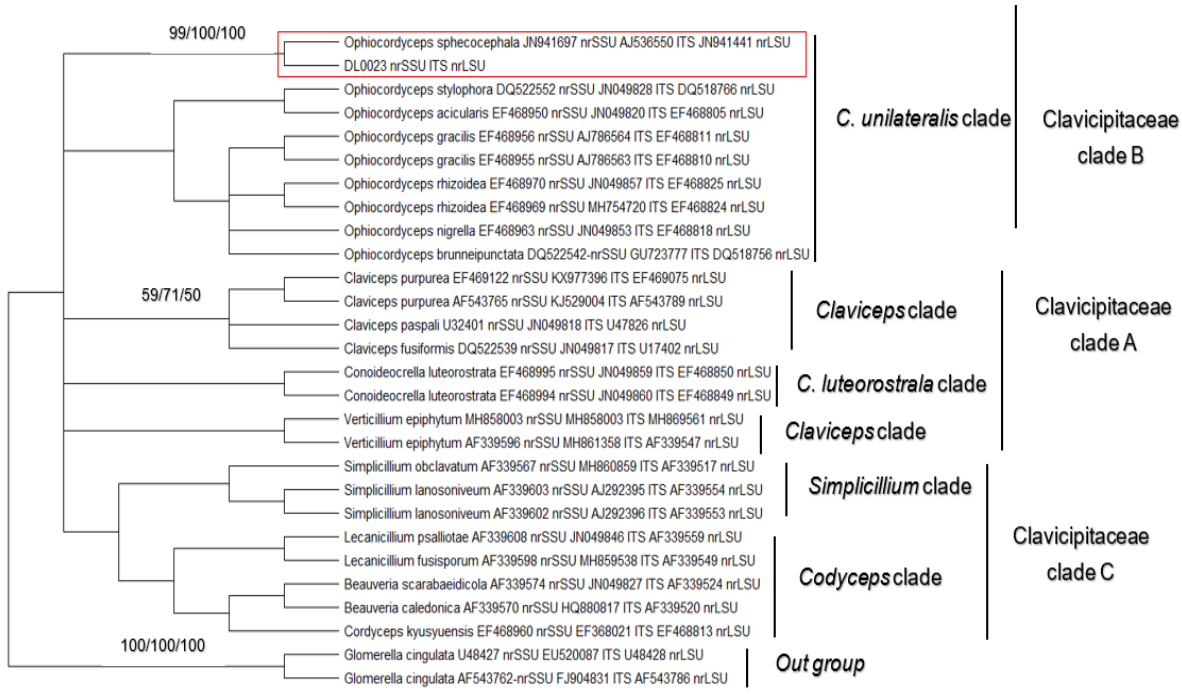



**Figure 3.** Phylogenetic trees of *ITS-nrLSU-nrSSU*: relationships among DL0023 and concatenated data set based on the analysis of ML method with bootstrap 1,000. Each internal branch's support values correspond to the NJ, MP, and ML methods

## 4. Discussion

During our journey to identify the variety of entomopathogenic fungus, the sample of DL0023 was taken at Lang Biang Biosphere Reserve, which has a great richness of both animals and flora because of the tropical monsoon environment with high temperatures and rainfall. To affirm the authenticity of DL0023, morphologically identified as *Ophiocordyceps sphecocephala*, the phylogenetic trees of *ITS*, *nrLSU*, *nrSSU*, and *ITS-nrLSU-nrSSU* were performed. It was noted that knowing the relationships among a particular fungal group has shown to be a beneficial goal for molecular phylogenetic methods for fungal evolution (Lücking & Hawksworth, 2018). Nowadays, the molecular data of *ITS*, *nrLSU*, and *nrSSU* were applied to support the species of in the kingdom of fungi. Herein, the multiple genes analysis of *ITS-nrLSU-nrSSU* was applied to strongly strengthen the identification of DL0023, resulting in forming with referent sequence of *Ophiocordyceps sphecocephala* within the highly supported bootstrap value (Bootstrap ≥ 99) and distinguishing from other referent taxon in subclades. Therefore, based on the phylogenetic analysis, the sample of DL0023 was identified as the *Ophiocordyceps sphecocephala*. Therefore, we concluded that the phylogenetic analyses based on the concatenated dataset successfully strengthened the identification of DL0023 as *Ophiocordyceps sphecocephala*, collected at Lang Biang Biosphere Reserve.

## 5. Conclusion

The phylogenetic analysis of multiple genes of *ITS*, *nrLSU*, *nrSSU*, were successfully identified the sample of DL0023, which was collected from Lang Biang Biosphere Reserve, as *Ophiocordyceps sphecocephala*.

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### Conflict of interest

The authors state no conflict of interest.

### Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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