DISCORVERY OF ENTOMOPATHOGENIC FUNGI *CORDYCEPS TAKAOMONTANA* AT LANGBIAN MOUNTAIN, LAM DONG, VIETNAM

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ABSTRACT

The stromata of Cordyceps takaomontana Y. Kobayashi (1941) were found on the Langbian Mountain, Da Lat, Vietnam at the height of 1.650 meter above sea level, on the larva of Lepidoptera. Stromata were lemon-yellow, clavate to enlongated clavate, arising from a white pseudosclerotium. The fertile head was on the top part of stromata, darker colored in comparison to the stipe. Perithecium was narrowly ovoid, superficial and forming dark yellow punctate on the surface of stromata. Ascus was cylindrical with semi-spherical cap. Ascospores were cylindrical, truncated and separately after discharge from the ascus.

Pure culture was isolated on Potato Glucose Agar (PGA) medium: white colony in young and yellow in old. The isolated mycelium was not homogenous in thickness and in growth rate at the peripheral area. Conidiophores were phialide, tapering to both apexes. Conidia had elliptical shape and formed into chains after maturation.

DNA was isolated, then purified from pure mycelium and used to amplifying the nrLSU (nuclear ribosomal large subunit) and rpb1 (RNA polymerase II largest subunit) genes. The amplified products were used for sequencing, proof-reading by some professional softwares before combining with other nrLSU and rpb1 sequences. Then this database was used to search for the suitable evolution model as well as to construct the phylogenetic trees.

The results of phylogenetic analysis completely supported the morphological classification: DL0038A and DL0038B were Cordyceps takaomontana Y. Kobayashi (1941).

Keywords: *Cordyceps takaomontana, Langbian, nrLSU, rpb1, Kobayashi.*

1. Introduction

The genus *Cordyceps*, emcompassing over 450 species, is the largest genus in the *Clavicipitaceae* family [7, 9]. The distribution is cosmopolitan, except Antarctica. In tropical and subtropical regions such as the Southeast and East Asia, the diversity is very high [7, 8, 9] including species that parasite on the larvae or the adult body of different insects.

Firstly described by Kobayashi in 1941, *Cordyceps takaomontana* parasitizes the larva of *Lepidoptera* [7]. According to the

classification of Kobayashi (1982) [7], *C. takaomontana* belongs to the subgenus *Eucordyceps*, *Cordyceps* genus, *Clavicipitaceae* family, *Hypocreales* order, *Sordariomycetes* class, *Pezizomycota* subphylum, *Ascomycota* phylum. *C. takaomontana* has pale yellow capitate stromata, fruiting body contains elliptical bowl-shaped parts that locate above the fertile part, forming dark yellow parts. Ascus is cylindrical with semi-spherical cap. Ascospores are cylindric, truncated and separate after discharge from the ascus [7].

The classification and identification of these fungi are mainly based on the analysis and dissection of morphology, which focuses on the structure of the fertile body, shape and size of ascus… However, due to the transformation between anamorphs and teleomorphs, the classification process has faced diverse difficulties. For example, *Tolypocladium inflatum* was firstly described in 1976 but it was not until 1996 that this species was defined as anamorphic state of *C. subessilus* by Hodge *et al.* [6]. Others factors that affect the classification process include the diverse species and the highly adaptive ability to environmental changes…

In 2007, Sung *et al.* combined different genes including *nrLSU* (nuclear ribosomal large subunit), *nrSSU* (nuclear ribosomal small subunit), *rpb1* (RNA polymerase II largest subunit), *rpb2* (RNA polymerase II second largest unit), *tub* (β-tubulin), *atp6* (mitochondrial ATP6) and *tef1* (elongation factor 1α) of 162 taxa to revise the genus *Cordyceps* and *Clavicipitaceae* family [14]. The results of this research clearly showed that the species belonging to this genus should be classified into three different family: *Claviciptaceae* family with *Metacordyceps*, *Hypocrella*, *Regiocrella* and *Torrubiella* genera, *Cordycipitaceae* family with *Cordyceps* genus and *Ophiocordycipitaceae* family with *Ophiocordyceps* and *Elaphocordyceps* genera [14], which has been acknowledged and used widely in many later researches.

nrLSU encodes the large subunit of ribosome (28S) that belong to the region IGS2-18S(SSU)-ITS1-5,8S-ITS2-28S(LSU)-IGS1-5S-IGS2 (IGS: intergenic spacer; ITS: internal transcribed spacer) [8]. The number of *nrLSU* in a cell is enormous due to the structure of rDNA containing various repeat units. Moreover, *nrLSU* contains both conserved and variable regions that are valuable in the support to identify the species [12, 14]. *rpb2* encodes for the largest subunit of RNA polymerase II [14]. This gene contains six conserved regions (A – H) that are also significant for phylogenetic analysis [13]. Therefore, both *nrLSU* and *rpb1* has been used widely to assist the identification of fungi [4, 14].

In the research fieldtrip to collect fungal samples in Langbian Mountain, Da Lat, we discovered 2 entomopathogenic species parasitizing on *Lepidopteran* larva (DL0038A and DL0038B). This research was then conducted to identify these samples in order to prove the diversity of the entomopathogenic fungi in the Highland in specific and Vietnam in general.

2. Methods and materials

2.1. Materials

The fungal samples DL0038A and DL0038B and insect body were collected at the Langbian Mountain, Da Lat and analyzed using light microscope.

Tissues of the fertile part of the fruit-body were applied to PGA (Potato Glucose Agar) medium to collect ascospores after 24 hours at 25 ± 2^{o}C. The size and shape of the ascopores were identified under microscope using Rax Vision (United States), then transferred to PGA petri disks for observation of anamorphic state.

2.2. Classification

The samples were classified under the methods of Y. Kobayashi (1982) [7] and Sung *et al.* (2007) [14].

2.3. DNA isolations

Pure mycelium was obtained from PGA culture of DL0038A and DL0038B. DNA was extracted using phenol/chloroform [2]. A

sterile metal stick was used to harvest the fungal mycelium (~1,5 g) then immersed into 700 µL of lysis buffer. The solution was incubated overnight at 65°C, centrifuged to remove insoluble compounds, and added 700 µL of PCI solution (Phenol/Chloroform/Isoamyl alcohol). DNA was isolated by centrifuge and collection of the upper liquid phase, and washed with Ethanol 70°. The extracted DNA was kept in TE solution at -20°C and was quantified using light spectrometry.

2.4. Polymerase chain reaction

The final volume of the reaction is 15 µL. PCR amplification was programmed as follow: 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 30 s, 55°C (*nrLSU*) or 46,3°C (*rpb1*) for 30 s, 72°C for 2 min, and 1 cycle of 72°C for 5 min. The primers were obtained from White *et al.* (1990) [17] and Sung *et al.* [14], including: LR0R-GTACCCGCTGAACTTAAGC (forward) and LR5-ATCCTGAGGGAAACTTC (reverse) for *nrLSU*, and CRPB1-CCWGGYTTYATCAAGAARGT (forward) and RPB1Cr-CCNGCDATNTCRTTRTCCATRTA (reverse) for *rpb1*. PCR products were electrophoresed on agarose gel (2%) and sequenced at Nam Khoa Company with the same primers.

2.5. Proofreading

DNA sequences were proofread to remove unclear signals at both ends, investigate the difference between forward and reverse primers and compare with database from Genbank (NCBI). Software include: Seaview 4.2.12 [5], Chromas Lite 2.1.1 [16], BLAST (Basic Local Alignment Search Tool – NCBI) [1].

2.6. Evolution model searching

78 sequences include representatives from *Clavicipitaceae* with *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk (*Glomerellaceae*) and *Verticillium ahlia* Kleb. (*Plectosphaerellaceae*) as outgroup. The database was homogenized with the final sequence length of 1552 bp. The data set was then used with jModelTest [3] to identify the most fit evolution model.

2.7. Phylogenetic analysis

Phylogenetic analysis was conducted by MEGA 6.0 [15]. To identify the incongruence of the dataset, bootstrap was applied with following characteristics: basic parameters were set to standard model, bootstrapping was repeated 1000 times with significant result when support value was over 50%, Tree bisection and reconnection (TBR) and MulTrees OFF.

3. Results and discussion

3.1. Morphological information

Teleomorphic state

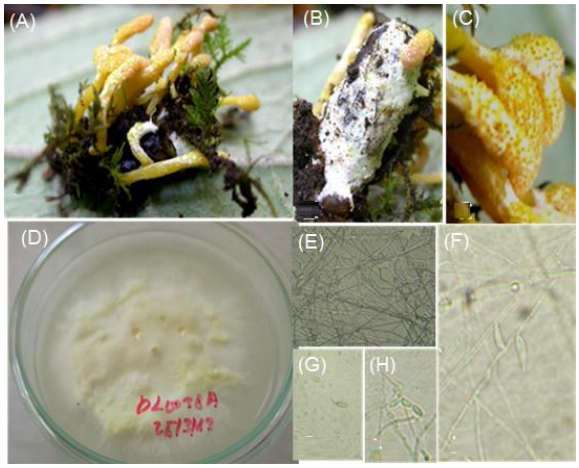
The two samples with more than 10 fruit bodies originated from individual or comprised stipitate. The fruit bodies (Fig 1A, 2A) were pale yellow, cylindrical or clavate, and formed from white pseudosclerotium (Fig 1B) with 1 – 5 cm in length and diameter of the fertile part up to 3,5 mm. The fertile part in development is covered with a white layer of sterile hyphae. Stipitate were pale yellow, smooth. The fertile head was on the top part of stromata, darker colored in comparison to the stipe. Perithecium was narrowly ovoid, superficial and forming dark yellow punctate on the surface of stromata (Fig 1C). Perithecium was narrowly ovoid (500 – 600 µm x 200 – 300 µm), superficial and forming dark yellow punctate on the surface of stromata. Mature asci arose from the cap to release ascospores. Asci were cylindrical (350 µm x 4 µm) with semi-spherical apical pore. Ascospores were cylindrical, truncated and separate after discharge from the ascus (4 – 5 µm x 1 µm).

Anamorphic state

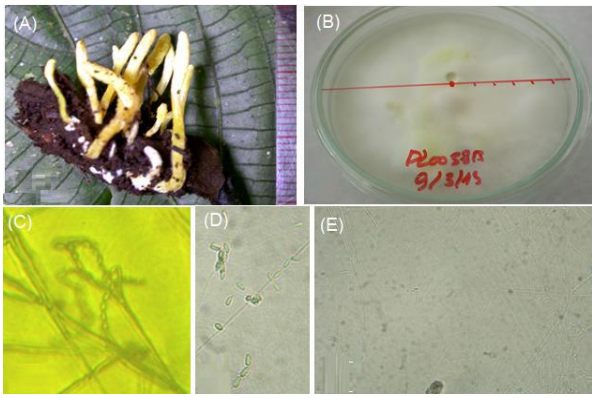
The mycelium developed well on PGA medium with high mycelium growth rate on PGA (Fig 1E). The color changed from white to yellow as the mycelium matured (Fig 1D, 2B). The isolated mycelium was not homogenous in thickness and in growth rate at

the peripheral area. Under light microscopy, conidiophores were phialide (Fig 1F, 2E), tapering to both apexes, 4 – 6 µm x 20 – 25 µm in size. Conidia had elliptical shape and formed into chains after maturation (Fig 1H, 2C, 2D) with 3 – 4 µm x 5 – 6 µm in size. Both samples were primarily classified as *C. takaomontana*.

Figurc 1. Morphology of DL0038A. A. Fruit-body; B: *Lepidopteran* host; C: Perithecium; D: mycelium on PGA medium; E: microscopic view of mycelium; F: Conidiophore; G: formation of conidia; H: mature conidia.



Figurc 2. Morphology of DL0038B. A: Fruit-body; B: mycelium on PGA medium; C, D: anamorphic spores; E: mycelium with conidiophores.



3.2. Phylogenetic analysis

DNA after extraction and purification from DL0038A and DL0038B (OD260/OD280 from 1,8 to 2,0) was amplified using PCR for *nrLSU* and *rpb1*. Electrophoresis on agarose gel 2% showed a clear band at 950 bp and 800 bp respectively (data not shown). PCR products were then sequenced at Nam Khoa Biotek Company with LR0R/LR5 primers and CRPB1/RPB1Cr primers and. Eight sequences (4 for forward primer and 4 for reverse primer) were proof-read. The sequences had clear peaks and no ambiguous peak was detected.

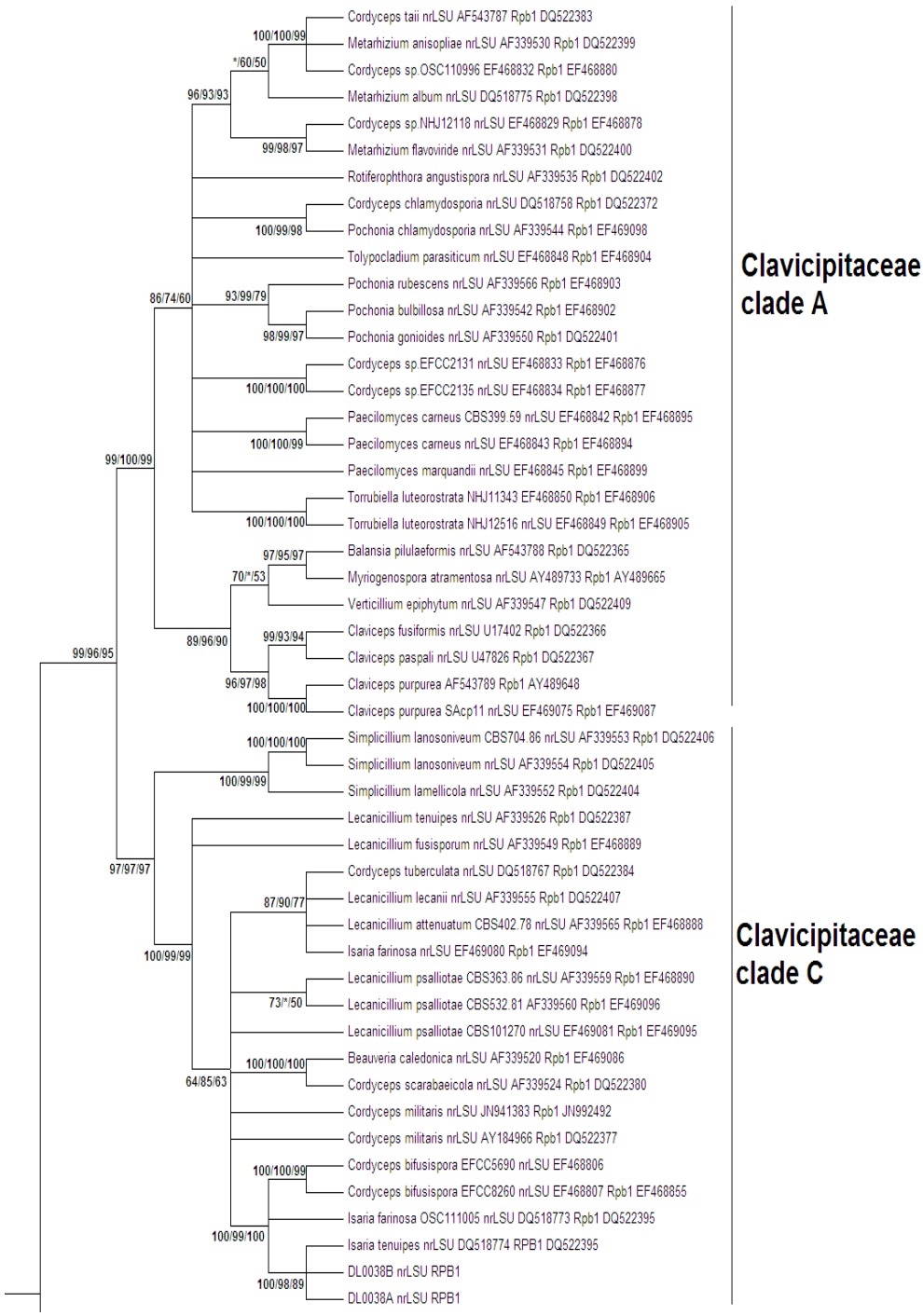
Database was set up accordingly to the data of Sung *et al.* (2007) [14]. The final set included 73 referent sequences, 2 for DL0038A and DL0038B and 3 for outgroup with average length of 1552 bp. Most probable evolution model was searched using jModelTest before construction of phylogenetic trees. The evolution model that is most fit with the observed data set was Jukes-Cantor, with following parameters: partition = 000000; -lnL = 29.939,17; P = 154.

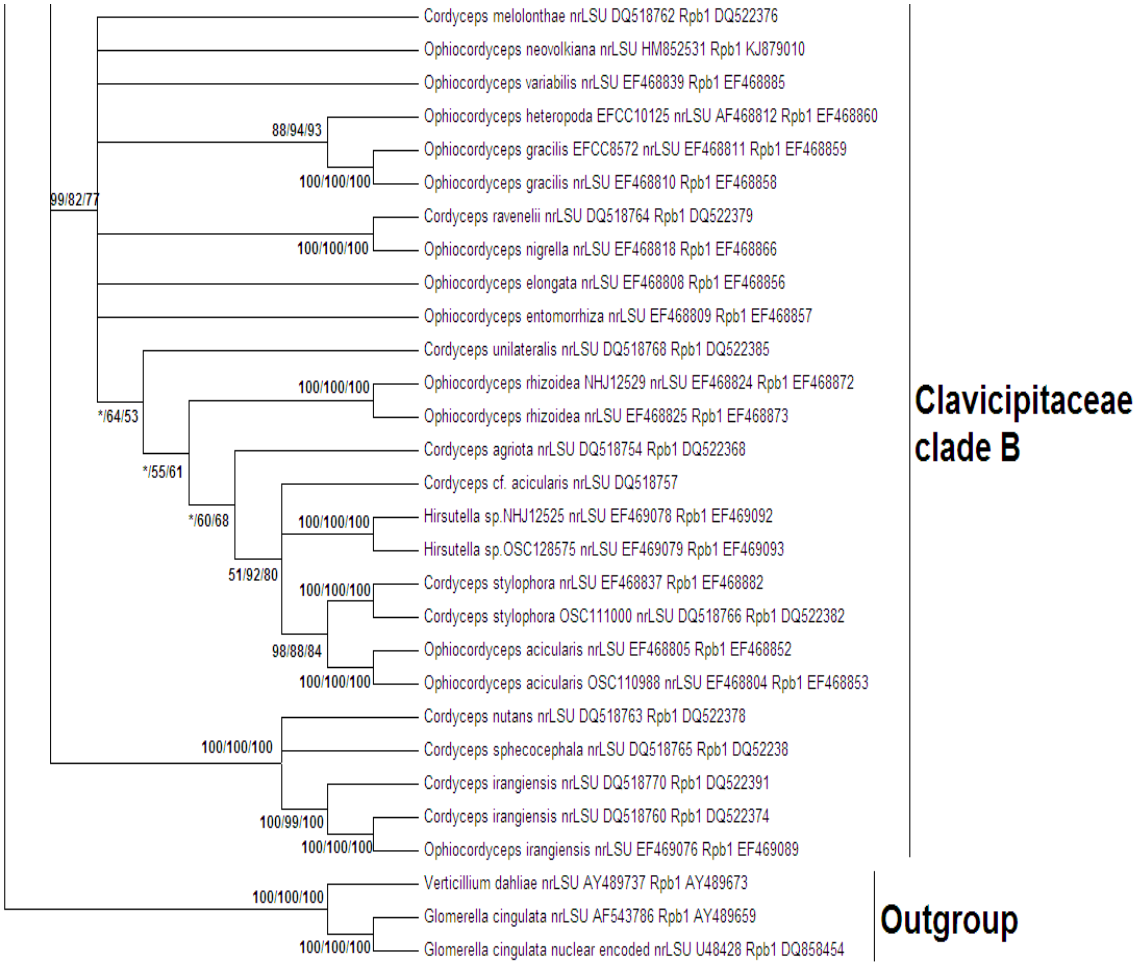
These parameters were applied to MEGA to construct phylogenetic trees, the results were:

The topology of neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) trees were similar to each other and to the trees from Sung *et al.* (2007) [13] (the shown phylogenetic tree is ML tree – Fig 3, with bootstrapping value of NJ/MP/ML trees on the branches). On ML tree (Fig 3.), 73 referent sequences from *Clavicipitaceae* with 26 sequences for clade B, 27 for clade A and 20 for clade C were all distributed logically and simlilarly to Sung *et al.* [14] and to outgroup. Bootstrapping values on these trees were all significant (Fig 3).

To be more specific, sequences of *Isaria tenuipes* (*nrLSU*: DQ518774; *rpb1:* DQ522395), DL0038A and DL0038B (Clade C) formed a monophyletic group with bootstrapping values (100/98/89) (highlighted in the red rectangular, Fig 3). *I. tenuipes* has been proven to be the anamorphic state of *C. takaomontana* [14]. Due to the limited number of sequences of this species on Genbank, only one referent was found for this species. This monophyletic clade was strongly supported and separated from other taxa in this clade including *C. bifusispora*, *C. militaris*, *C.*

scarabaeicola (*Cordyceps* genus), *Beauveria caledonica* (*Beauveria* genus), *Isaria farinosa* (*Isaria* genus), *Lecanicillium tenuipes*, *L. attenuatum*, *L. psalliotae*, *L. fusisporum* and *L. lecanii* (*Lecanicillium* genus), and *Simplicium lamellicola*, *S. obclavatum*, *S. lanosoniveum* (*Simplicium* genus). Moreover, this monophyletic group was highly supported by bootstrapping values when compared with other taxa of clade C. such as *C. bifusispora* and *I. farinosa* (100/99/99). These results clearly showed that the phylogenetic analysis was completely in agreement with morphological analysis and that DL0038A and DL0038B belong to *C. takaomontana* in its teleomorphic state and *I. tenuipes* in its anamorphic states.





Figurc 3. Phylogenetic tree from *nrLSU* and *rpb1*. Maximum likelihood tree is shown with bootstrapping value of NJ/MP/ML. An asterisk (*) implies a bootstrapping value under 50 or ambiguous branches between trees.

4. Conclusion

We have successfully applied the primary identification using morphology and phylogenetic analysis basing on *nrLSU* and *rpb1* to assist the classification of 2 samples from entomopathogenic fungi (DL0038A and DL0038B) obtained from Langbian Mountain, Da Lat and concluded that these two samples are *Cordyceps takaomontana*.

This research will be further developed using other multigenic phylogenetic analysis (*nrSSU, rpb1, rpb2* and *tef1*) to enhance the support of the classification method and also applied this method to identify other samples from our collection.

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