Journal of Science and Technology ***55*** *(1A) (2017) 19-26*
DOI: 10.15625/2525-2518/55/1A/12378

DISCOVERY OF ENTOMOPATHOGENIC FUNGI *Cordyceps takaomontana* AT LANGBIAN MOUNTAIN, LAM DONG, VIET NAM

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Received: 30 October 2016; Accepted for publication: 30 May 2017

ABSTRACT

The stromata of *Cordyceps* sp. 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 elongated clavate, arising from a white pseudosclerotium. The fertile head was on the top part of stromata, darker coloured in comparison to the stipe. Perithecium was narrowly ovoid, superficial and forming dark yellow punctate on the surface of stromata. Ascus cylindrical with semi- spherical cap. Ascospores were cylindric, 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 form into chains after maturation.

DNA was isolated, then purified from pure mycelium and used to amplifying the *nrLSU* (nuclear ribosomal large subunit) sequence. The amplified products were used for sequencing, proof-reading by some professional solfwares before combining with other *nrLSU* 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*.

Keywords: *Cordyceps takaomontana*, Langbian, Mega 6.0, *nrLSU*, Kobayashi.

1. INTRODUCTION

Cordyceps comprises of more than 450 species and is the most diverse genus among Clavicipitaceae [1, 2]. *Cordyceps* species are found worldwide except Antartica. The diversity is the highest at tropical and subtropical areas like the Southeast and East Asia [1, 2, 3] with host range from larvae to mature insect species. Moreover, the host can also be non-insect species including fungi of *Elaphomyces* [4]. Several *Cordyceps* sp. are well-known for their use in traditional medicine among Asian countries including Vietnam [5].

The stromata of *Cordyceps takaomontana* was first identified by Kobayasi in 1941 which paratisize on Lepidopteran larvae. According to his classification system [1], *C. takaomontana* belongs to the subgenera *Eucordyceps* (*Cordyceps*, Clavicipitaceae, Hypocreales, Sordariomycetes, Pezizomycota, Ascomycota) with fertile part containing multiple superficial ovoid perithecia which create dark yellow spots on the part, asci cyclindral with apex apices, ascospore cylindral, often disarticulate into part-spores upon maturation [1].

The classification and identification of *Cordyceps* fungi mainly depend on morphological criteria which focus on the structure of the fertile part and size and shapes of asci. Moreover, the existences of a binominla nomenclature causes tremendous troubles for the identification process. A typical example of this problem is the case of *Tolypocladium inflatum* which was identifed in 1976. However, it was not until 1996 that Hodge et al. discovered that this fungi is the anamorphic state of *Cordyceps subsessilus* [6]. Morever, other factors such as intraspecies diversity, the ability to adapt to environmental changes, etc. do contribute in complicating the whole process.

In 2007, Sung et al. combined several gene sequences including *nrLSU* (nuclear ribosomal large subunit), *nrSSU* (nuclear ribosomal small subunit), *rpb1* (largest subunit of RNA polymerase II), *rpb2* (second largest subunit of RNA polymerase II), *tub* (β tubulin), *atp6* (mitochondrial ATP6) and *tef1* (the elongation factor 1α) of 162 taxon to reclassify the systematics of *Cordyceps* and Clavicipitaceae [7]. From this publication, *Cordyceps* fungi are now divided into 3 different families, namely Clavicipitaceae (with *Metacordyceps*, *Hypocrella*, *Regiocrella*, and *Torrubiella*), Cordycipitaceae (with *Cordyceps*), and Ophiocordycipitaceae (with *Ophiocordyceps* and *Elaphocordyceps*) [7]. This classification is considered to be basic for all research being done on *Cordyceps.*

nrLSU encodes for the large subunit of ribosome and belongs to a repetitive unit of rDNA : IGS2-18S(SSU)-ITS1-5.8S-ITS2-28S(LSU)-IGS-5S-IGS2 (IGS: Intergenic spacer, ITS: Internal transcribed spacer, SSU: ribosomal small subunit) [7]. The number *nrLSU* copy within a cell is enormous compared to other nuclear genes. Moreover, *nrLSU* contains highly conserved regions in between variable ones (D1, D2, D3) whose information is valuable in assisting the identification to species level [7, 8]. Therefore, *nrLSU* has been widely used as a marker for fungi identification such as Dentinger et al. [9] and Sung et al. [7].

On different field trips to collect fungi samples in Langbian Mountain, Lam Dong Province, Vietnam, we have discovered two entomopathogenic fungi on Lepidopteran host (DL0038A and B). This research was conducted to identify these samples to assess the diversity of entomopathogenic fungi in the Highlands of Vietnam.

2. MATERIALS AND METHODS

2.1. Fungal sample

Two entomopathogenic samples (DL0038A and B) with host remains found at the mountainous region of Langbian, Dalat were analyzed in a laboratory with optical microscopy (Rax Vision, USA).

A small tissue from the fertile part of the stromata was obtained and pasted on the surface of a petri disk containing PGA media to obtain ascospore in 25 ± 2 ^{o}C for 24 h. The morphology and budding of ascospores were observed under optical microscopy. The samples were then subcultured on PGA disks for anamorphic identification.

2.2. Morphological identification

The samples were identified based on Y. Kobayashi [1] and Sung et al. [7].

2.3. DNA isolation

DNA was isolated from the mycelia on PGA disks. The process was conducted according to Chomczynski & Sacchi [10] with the assistance of Phenol/Chloroform. Firstly, mycelia was collected by a sterile stem and transfered into a tube containing lysis buffer. The mixture was incubated overnight at 65 ^{o}C and centrifuged to collect the supernatant. 700 µL of PCI (Phenol/Chloroform/Isoamylalcohol) solution was added and centrifuged. The upper solution was collected, precipitated with absolute ethanol, and washed with 70 % enthanol. DNA concentration was identified by using OD260. The samples were kept in TE buffer at -20 ^{o}C.

2.4. PCR

The final volume for PCR was 15 µL with a specified program: 1 cycle of 95 ^{o}C for 5 min; 40 cycles of 95 ^{o}C in 30 s, 55 ^{o}C in 30 s. 72 ^{o}C in 2 min; 1 cycle of 72 ^{o}C in 5 min. The primers were LR0R 5'-GTACCCGCTGAACTTAAGC-3' and LR5 5'-ATCCTGAGGGAAACTTC-3' [11]. The amplified product was sequenced at Nam Khoa Company with the same primers.

2.5. Sequence proofreading

DNA sequences were proofread to remove ambiguous signals at both ends. The sequences were then blasted on GenBank (NCBI). The softwares used for proofreading include SeaView 4.2.12 [12], Chromas Lite 2.1.1 [13] BLAST (NCBI) [14].

2.6. Model of substitution

63 sequences of Clavicipitaceous fungi with *Glomerella cingulata* (Glomerellaceae) and *Verticillium dahliae* (Plectosphaerellaceae) as outgroup (Datasize is 691 bp) were analyzed by jModelTest [15] to identify the best fit model of substitution.

2.7. Phylogenetic analysis

Phylogenetic tree was constructed with MEGA 6.0 [16] with a 1000 replicate boostrap. The tree was searched by TBR mode with MulTrees OFF.

3. RESULTS AND DISCUSSIONS

3.1. Morphological identification

3.1.1. Telemorphic

The two samples have more than 10 indenpendent stipitates, fleshy and pliant. Stipitate (Figures 1 and 2) pale yellow, cylindrical or clavate was formed on white pseudosclerotium (Figure 1B) with 1 - 5 cm in length and diameter of the fertile part up to 3.5 mm. Immature fertile part is covered in layers of white anamorphic ascospores. The stem of the stipitate is pale yellow, smooth. Fertile part is located near the tip of the stipitate, dark yellow with perithecia partly superficial on the surface creating dark yellow spots on the part (Figure 1C). Upon maturation, asci are released from pertithecia. Perithecia is ovoid, 500 - 600 µm × 200 - 300 µm. Ascus is cylindrical (350 µm × 4 µm) with apex apices. Ascospores are cylindrical and often disarticulate into part-spores upon release, 4 - 5 µm × 1 µm.

3.1.2. Anamorph state

The mycellium developed on PGA medium with high growth rate (Figure 1E). The color changed from white to yellow was the mycellium matured (Figures 1D, 2B). The isolated mycellium was not homogenous in thickness and in growth rate at peripheral areas. Under light microscopy, conidiophores were phialide (Figures 1F, 2E), tapering to both apexes, 4 - 6 µm × 20 - 25 µm in size. Conidia had elliptical shape and formed into chains after meturation (Figures 1H, 2C, 2D), 3 - 4 µm × 5 - 6 µm. Both samples were considered to be *Cordyceps takaomontana*.

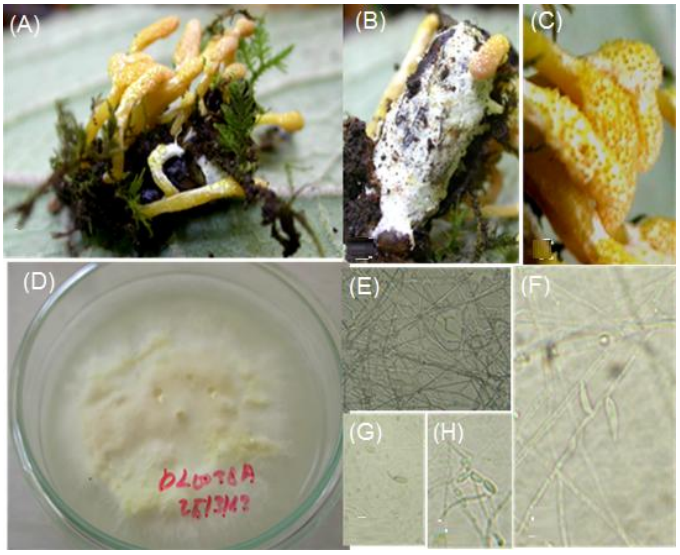

Figure 1. Morphology of DL0038A. A. Stromata; B. Lepidopteran host; C. Perithecium; D. Mycellium on PGA medium; E. Microscopic view of mycellium; F. Conidiophore; G. Conidia; H. Mature conidia.

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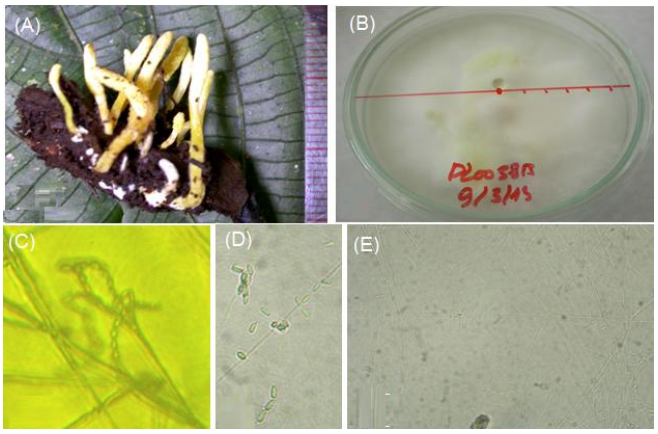

Figure 2. Morphology of DL0038B. A: Stromata; B: Mycellium on PGA medium; C, D: Anamorphic spores; E: Mycellium with conidiophores.

3.2. Phylogenetic analysis

DNA after extraction and purification was amplified with LR0R and LR5 primers. Electrophoresis on 2 % agarose gel showed a clear band at 950 bp. PCR products were sequenced at Nam Khoa Company. The sequences had clear peaks, were proofread and blasted on NCBI. Database was set up accordingly to Sung et al. [7]. The final dataset containing 57 referent taxon, 3 outgroup and the 2 samples was 698 bp in length and was analyzed by jModelTest. The best fit model was the TN93 + G model.

The parameters were input onto MEGA software to construct phylogentics trees. The topology of Neighbor-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) trees were similar with no major conflict. An ML tree is shown here with boostrap value of NJ/MP/ML on each branch (Figure 3). This tree contains 26 taxon from clade B, 12 from clade A and 19 from clade C. All of the taxons are distributed accordingly to Sung et al. [7] publication. Bootstraping values on these clades were all significant.

To be more specific, in the formation of clade C, the sequences of DL0038A and DL0038B formed a monophyletic group with two referent sequences of *C. takomontana* (ID: AB044637, KC610756) with boostrap value of 75/69/73 % and separated this group from other referent taxon including *C. bifusispora*, *C. militaris*, *C. scarabaeicola*, *Beuveria caledonica*, *Isaria farinosa*, *Lecanicillium tenuipes*, *L. attenuatum*, *L. psalliotae*, *L. fusisporum*, *L. lecanii*, *Simplicium lamellicola*, *S. obclavatum* and *S. lanosoniveum* (Figure 3). Thus, the result of phylogentic analyses were in concordance with the morphological analysis. DL0038A and DL0038B belong to *Cordyceps takaomontana.*

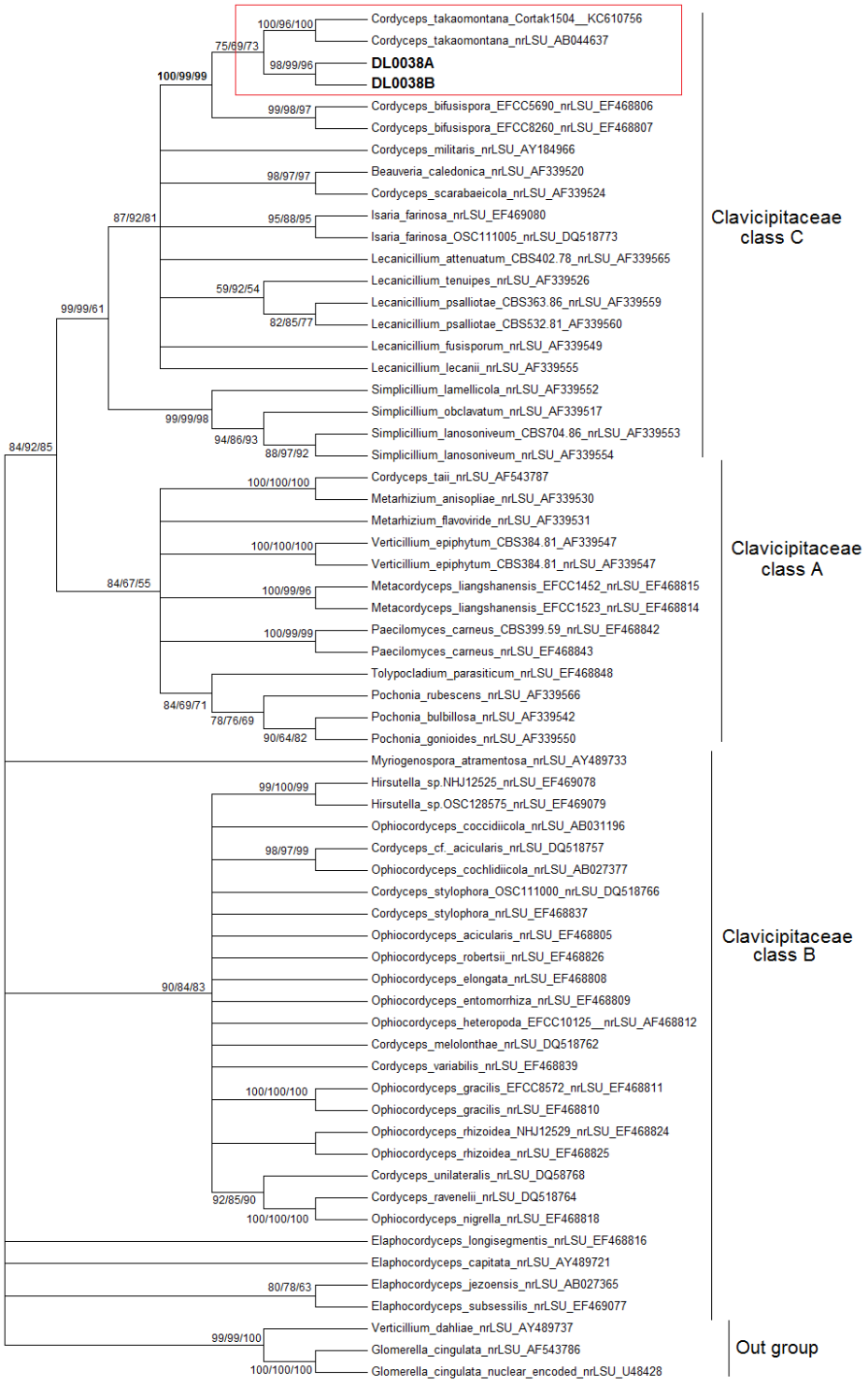

Figure 3. Phylogenetic tree from *nrLSU* sequences with boostrap values on each branch.

4. CONCLUSION

We have successfully applied the identification based on morphology and phylogenetics relying on *nrLSU* sequence to analyze the samples of entomopathogenic fungi (DL0038A and DL0038B) from the mountainous region of Langbian, Dalat and concluded that these samples are *Cordyceps takaomontana*.

This research will be continued with the analysis based on multigen phylogenetics to increase the support of molecular data. Moreover, this identification procedure will be applied for the other samples in our collection.

Acknowledgement. This research was conducted on the support of the Ho Chi Minh City Department of Science and Technology and the Young Scientist Program 2014 – 2015 for Lao Duc Thuan (MSc).

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