Journal of Science and Technology ***55*** *(1A) (2017) 93-98*

DOI: 10.15625/2525-2518/55/1A/12385

ANALYSIS OF *nrLSU* GENE TO SUPPORT IDENTIFICATION OF FUNGUS BELONGING TO *Cordyceps* GENUS AND *CLAVICIPITACEAE* FAMILY

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

ABSTRACT

Nucleotide sequences of the nuclear large ribosomal subunit (*nrLSU*) have been used in fungal systematics for a long time. *nrLSU* was also used in *Cordyceps* and related genera within the *Clavicipitaceae* family. A previously identified sample by morphology and ITS was used in this research to analyze the ability of *nrLSU* to support the identification of entomopathogenic fungi. Our results show that we successfully amplified *nrLSU* gene using the primer pair LR0R and LR5. The PCR product on agarose gel showed a clear band at 950 bp. Sequencing method was then adopted and proofread before molecular phylogenetic analysis was applied with reference sequences obtained from the publication of Sung et al. Once again, this analysis confirms the DL0004 specimen as *Cordyceps neovolkiana*.

Keywords: Cordyceps, Cordyceps neovolkiana, nrLSU, molecular phylogenetics.

1. INTRODUCTION

According to our previous publication [1], the fungal specimen DL0004 was found on rotting leaves, under neotropical forest at the height of 1650 meters at LangBiang Mountain, Lam Dong Province. The fungus has yellow clavate fruitbody with two clear part. The fertile part is pale yellow with clavate head and is 2-3 mm wide and 3-5 mm long (Figure 1a). The stroma is yellow and fibrous. The fungus has cylindrical perithecia with thickened wall that is superficious on stromata surface ranging 350-400 µm with parallel asci (Figure 1b). Upon maturation, the perithecia opens to release asci containg ascospores. The asci is filiform with sharp ending at the bottom of perithecia and thinkened ascus apices, ranging 250-300 µm × 7-9 µm in size (Figure 1c). Each asci contains five ascospores. Ascospore is filiform, separating into

three part-spores, with thickened wall, ranging 4-5 µm × 2-3 µm in size (Figure 1d). These characteristics correspond to the descriptions of Kobayasi [2] for *Cordyceps neovolkiana*.

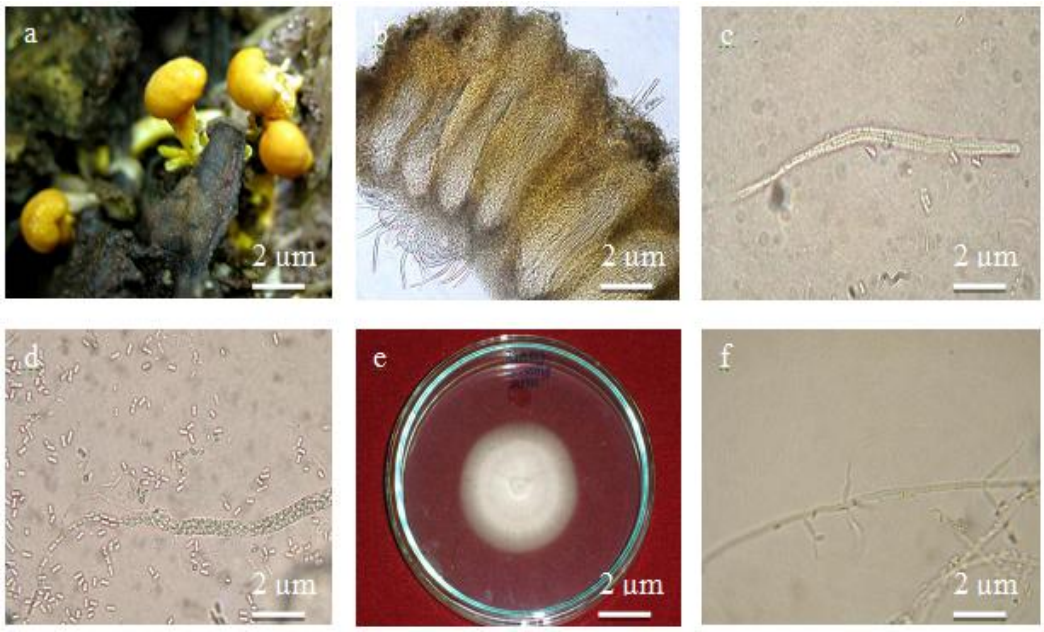

Figure 1. Morphology of DL0004. a. stromata in natural environment; b. perithecia; c. ascus; d. ascospores; e. colony on PGA medium; f. conidiophores.

ITS rDNA region, sequenced and analyzed with 17 sequences from different species of *Cordyceps* (NCBI) using molecular phylogenetics, showed that DL0004 was *Cordyceps neovolkiana* [3].

In recent years, several publications have shown the benefits of using multigenes in assisting the identification of fungi belonging to the entomopathogenic genus. Chan et al. analyzed the *ITS*, *nrLSU*, *EF-1α*, *rbp1* to assist the identification of *C. gunnii* in China [4]. Johnson et al. utilized *nrSSU*, *nrLSU*, *tef*, *rpb1*, *rpb2* to analyze fungi belonging to *Torrubiella* genus [5]. Park et al. conducted phylogenetic analysis on ITS1-5,8S-ITS2 for *Cordyceps* that parasitize butterflies [6]. Here, the publication of Sung et al. is particularly interesting [3] since the relationship between *Clavicipitaceae* family and *Cordyceps* genus were reclassified. The research focused on 5-7 regions belonging to the house-keeping genes group including *nrSSU* (nuclear ribosomal small subunit), *nrLSU* (nuclear ribosomal large subunit), *tef1* (elongation factor 1α), *rpb1* (largest subunit of RNA polymerase II), *rpb2* (second largest subunit of RNA polymerase II), *tub* (β tubulin), and *atp6* (mitochondrial ATP6) of 162 taxons. The result showed similarities between morphological and molecular identifications. *Cordyceps* were reclassified into three families including *Clavicipitaceae* (*Metacordyceps*, *Hypocrella*, *Regiocrella*, *Torrubiella*), *Cordycipitaceae* (*Cordyceps*), and *Ophiocordycipitaceae* (*Ophiocordyceps*, *Elaphocordyceps*). The dataset of the publication was also the largest up to now on Genbank (NCBI).

In order to conduct multigene analysis, separate gene data must be analyzed first to test incongruence and notice the difference between datasets [3]. In this current research, *nrLSU* was

used as a first step towards the formation of a multigene dataset. 162 sequences from Sung et al. [3] of *nrLSU* were analyzed for the fungal specimen DL0004.

2. MATERIALS AND METHODS

2.1. Fungus sample

Cordyceps neovolkiana Y. Kobayashi 1941 mycelium was supported by the Faculty of Biology, Da Lat University.

2.2. DNA isolation and PCR

DNA was isolated from the mycelia on PGA disks. The process was conducted according to Chomczynski & Sacchi [7] with the assistance of Phenol/Chloroform. Firstly, mycelia was collected by a sterile stem and transferred into a tube containing lysis buffer. The mixture was incubated overnight at 65 °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 % ethanol. DNA concentration was identified by using OD260. The samples were kept in TE buffer at -20 °C.

The final volume for PCR was 15 µL with a specified program: 1 cycle of 95 °C for 5 min; 40 cycles of 95 °C in 30 s, 55 °C in 30 s. 72 °C in 2 min; 1 cycle of 72 °C in 5 min (Table 1) [8]. The amplified product was sequenced at Nam Khoa Company with the same primers.

Table 1. Primers for *nrLSU.*

Gene region	Primers		Sequence (5’ – 3’)	Size
nrLSU [9]	F	LR0R	GTACCCGCTGAACTTAAGC	950 bp
	R	LR5	ATCCTGAGGGAAACTTC	

2.4. Sequence proofreading, Phylogenetic analysis

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, Chromas Lite 2.1.1, BLAST (NCBI). Sequences of Clavicipitaceous fungi with *Glomerella cingulata* (Glomerellaceae) and *Verticillium dahliae* (Plectosphaerellaceae) as outgroup were analyzed by jModelTest to identify the best fit model of substitution. Phylogenetic tree was constructed with MEGA 6.0 with a 1000 replicate bootstrap. The tree was searched by TBR mode with MulTrees OFF [9].

3. RESULTS AND DISCUSSION

DNA after extraction and purification was amplified with LR0R and LR5 primers. Electrophoresis on 2 % agarose gel showed a clear band at 950 bp (Figure 2). PCR products were sequenced at Nam Khoa Company. The sequences had clear peaks (Figure 3), were proofread and blasted on NCBI. Database was set up accordingly to Sung et al. [3]. The final

dataset was established based on Sung et al. [3] and BLAST results. Phylogenetic trees were constructed by MEGA 6.0.

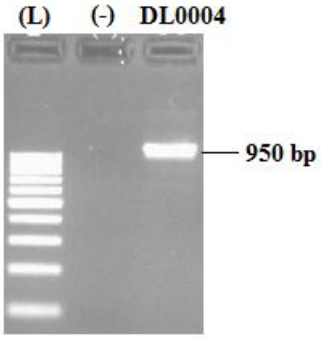

Figure 2. Electrophoresis for PCR product of nrLSU. (L): 100bp Ladder, (-): negative control, and DL0004.

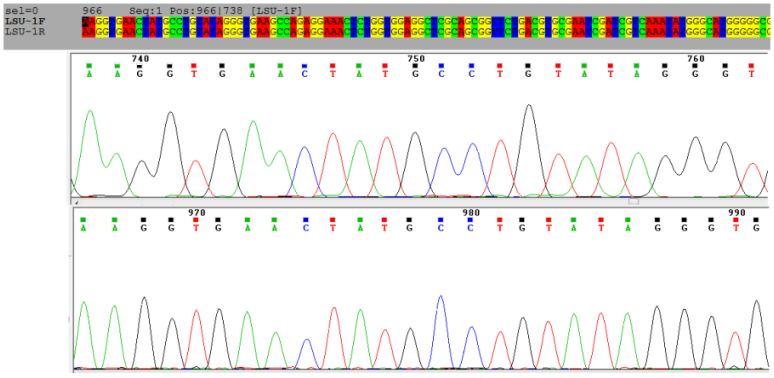

Figure 3. A part of the sequences from the forward and reverse primers.

The topology of Neighbor-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) trees were similar with no any conflict, therefore, an ML trees which was integrated NJ, MP into ML, was shown here with bootstrap value of NJ/MP/ML on each branch of tree (Figure 4). Molecular phylogenetic analysis separated clade A, B and C with outgoup. The formation of clade A, B, C was almost identical between current result and Sung et al. [3], except for some referent sequences of Clade A (Figure 4). However, reference sequences of Clade B including DL0004 showed the suitable relationship (Figure 4), especially DL0004 formed a monophyletic group with several Clade B entomopathogenic fungi such as *Cordyceps nutans* (DQ518763), *Ophiocordyceps tricentri* (AB027376), *Cordyceps sphecocephala* (DQ518765), and *Cordycep irangiensi*s (DQ518770, DQ518760, EF469076) (Figure 4). Within this clade, DL0004 formed a highly supported monophyletic group with two reference sequences *Ophiocordyceps neovolkiana* (KJ878896) and *Ophiocordyceps melolonthae* (DQ518762) with bootstrap values 100, 100 and 90 for Neighbour Joining, Marximum Parsimony, Marximum Likelihood methods, repectively, and separated this group from other reference taxon (Figure 4). Combining with morphological identification, the molecular identification showed similar result as *Cordyceps neovolkiana*. Moreover, according to Sung et al. [3], *Cordyceps neovolkiana* [2] is *Ophiocordyceps neovolkiana* [10]. Therefore, from these results, we could conclude that DL0004 is *Cordyceps neovolkiana*.

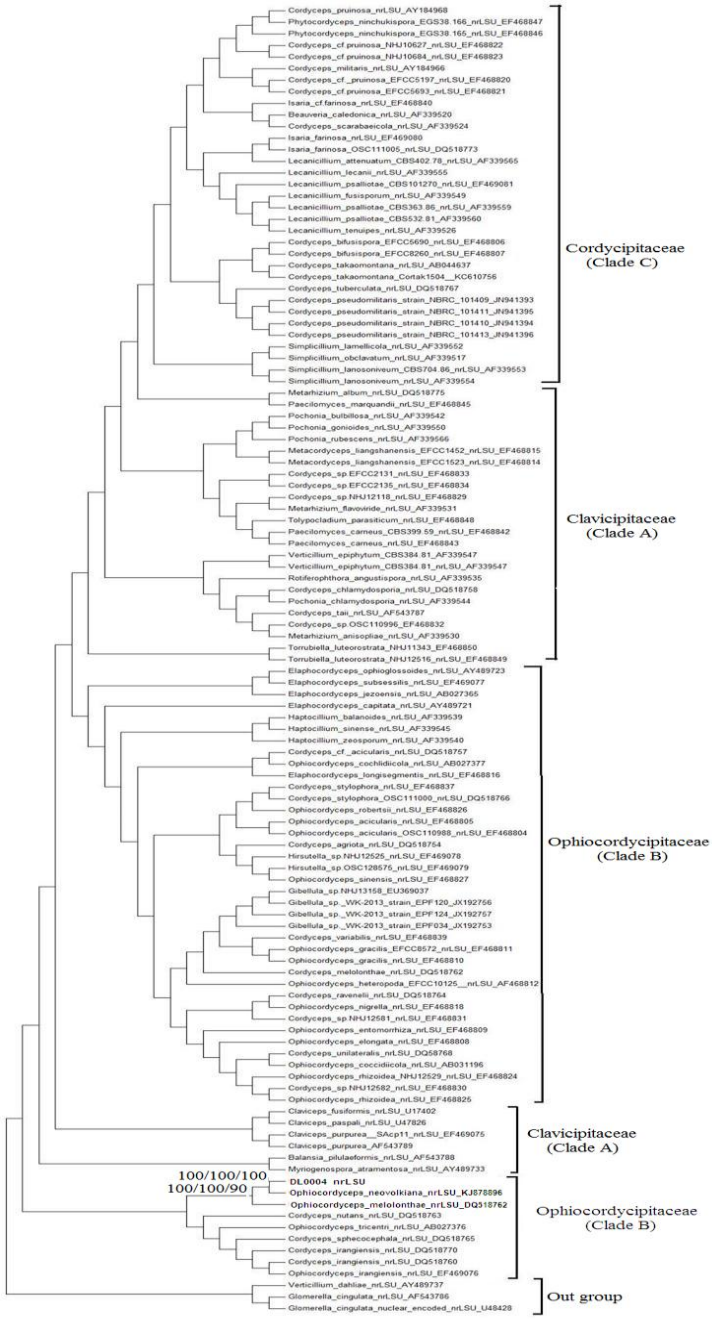

Figure 4. Molecular phylogenetics of *nrLSU* by Maximum Likelihood with bootstrap proportions of NJ/MP/ML methods.

This result was also identical to the work of Le et al. [1] on *ITS* region that also showed DL0004 to be *Cordyceps neovolkiana.* Therefore, the result of this research confirmed the ability of molecular phylogenetics to assist the identification of entomopathogenic fungi.

4. CONCLUSION

We have successfully applied molercular biology in combination with bioinformatics for *nrLSU* to assist the entomopathogenic sample DL0004. The resolution of the analysis process was to the species level and identical with *ITS* analysis. Moreover, this methodology can be applied for other samples of the 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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