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EFFICIENCY OF ITS1-5.8S-ITS2 REGION IN IDENTIFYING CORDYCEPS SPECIES

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SUMMARY

Cordyceps genus is a well-known traditional medicine worldwide. It contains abundant physiological active compounds that were demonstrated to perform benefit in reducing progression of cancer as well as protecting human health. Accurately classifying species in this genus is essential in order to prevent commercial counterfeit medicines. Nowadays, a taxonomic classification of species based on DNA sequences can overcome the existed limitation in identifying by using only morphological characteristics of this genus. DNA barcodes are standard short genomic regions that are universally present in target lineages and has sufficient sequence variation to discriminate species in the genus. A variety of loci has been suggested as DNA barcodes for plants, including genes and non-coding regions in the nuclear and plastid genomes such as *psb*A-*trn*H, *mat*K, *rbc*L, and ITS. Thus, the objective of this study was to identify selected species of *Cordyceps* genus using DNA barcodes. Seven strains of *Cordyceps* were collected. Total DNA extraction and purification, PCR amplification and DNA sequencing were performed with standard chemicals and kits. The candidate ITS1-5.8S-ITS2 region was amplified and sequenced. Data were analyzed using Bioedit 7.2.6 and MEGA 7 softwares. Analysis of seven obtained DNA barcode sequences of collected samples revealed that the ITS1-5.8S-ITS2 region provided high species discriminating power for *Cordyceps* genus. Accordingly, phylogenetic trees based on this DNA barcode exhibited six samples had closed relationship to *Cordyceps militaris*, while another specimen was the nearest neighbor to *Cordyceps sinensis* with average similarities at 99.82% and 99.81%, respectively. Our results support the identification of valuable medicinal plant species within *Cordyceps* genus.

Keywords: *Cordyceps militaris*, *Cordyceps sinensis*, ITS1-5.8S-ITS2 region, DNA barcode, species discrimination

INTRODUCTION

Genus *Cordyceps* belongs to division Ascomycota, class Sordariomycetes, order Hypocreales, family Clavicipitaceae and is entomopathogenic and endoparasitic fungi (Sung *et al*., 2007). *Cordyceps* species mainly parasitize in arthropod from lepidopteran larva and pupae to imago. They are distributed predominantly in higher than 4000 meter mountainous areas of Asia such as the Tibetan Plateau and Himalayan Mountain Range. They thrive vigorously in hot and humid subtropical climate and temperate forests. Currently, approximate 400 species of this genus have been described (Mains, 1957; Kobayasi, 1982; Stensrud *et al*., 2005). Many species of *Cordyceps* have been used as health food or medicine in China and South-East Asia since it contains abundant physiologically active compounds (Masuda *et al*., 2007; Hur, 2008). According to modern medicine, there are seven groups of basic organic compounds of *Cordyceps*: (1) Proteins, peptides, essential amino acids; (2) Polysaccharides; (3) Sterol including ergosterol, Delta-3 ergosterol, ergosterol peroxide, 3-sitosterol, daucosterol and campeasterol; (4) Nucleosides (also known as cordycepins) include adenine, uracil, uridine, guanosine, thymidine; (5) Saturated and unsaturated fatty acids; (6) Vitamins (B1, B12, E, K); (7) Minerals (K, Na, Ca, Mg, Fe, Cu, Mn, Zn, Pi, Se, Al, Si, Ni, Sr, Ti, Cr, Ga, V, and Zr). The main uses of *Cordyceps* have been demonstrated through a range of studies. Crude extracts of *Cordyceps sinensis* enable to inhibit the growth of several cancer cell linages such as K562, Vero, Wish, Calu-1

and Raji (Kuo *et al*., 1994). *Cordyceps* acts on the immune system in two different ways, both boosting the immune system of people with immunodeficiency in cancer, diabetes, cardiovascular disease, while reducing unwanted immune responses for people with increased immunity (leukemia and organ transplantation, for instance) (Feng *et al*., 2008; Jordan *et al*., 2008; Ding *et al*., 2009). They also enhanced digestion and absorption by acting on the intestinal microflora (Koh *et al*., 2003). For glucose regulation, Lo *et al*. (2004) observed that diabetic mice fed with *C. sinensis* in 28 days gained weight and decreased blood glucose level compared to the mice using placebo (Lo *et al*., 2004). Adenosine in *Cordyceps* showed useful in protecting heart health, increase circulation and preventing cardiac arrhythmia (Mei *et al*., 1989), ischemia, and stroke (Liu *et al*., 2010). *Cordyceps* has a positive influence in sperm formation and restore sexual dysfunction in both genders (Zhu *et al*., 1998; Zhang *et al*., 2005). By reducing risk of Alzheimer disease due to β-amyloid *Cordyceps* proved to improve concentration and memory (Ji *et al*., 2009; Jin *et al*., 2004). Function of *Cordyceps* in improving liver function were showed in increasing ALT in hepatitis B virus infection patients (Zhu *et al*., 1992; Gong *et al*., 2000). *Cordyceps* also help to inhibit renal fibrosis, protect the kidneys from the effects of cyclosporine A (Wu *et al*., 2000). Such effects made *Cordyceps* to become a valuable tradition medicine not only in Asia but also in Western countries and at risk of commecial fraud due to very high price. Currently, besides the morphological classification, molecular techniques had been applied to increase accuracy and reliability identification of mushroom *Cordyceps* in order to control trading activities of this genus.

DNA molecular barcodes based on short DNA fragments in mitochondrial genome for animals or in chloroplast and nuclear genomes for plants were developed to discriminate species. Mitochondrial DNA is an extranuclear genetic material which is capable of replicating independently with the nuclear DNA. Since mitochondrial DNA evolves five times faster than nuclear genetic markers, it becomes an effective tool for evaluating genetic diversity in phylogenetics and evolutionary biology. However, the rate of evolution of the mitochondrial genome in plants is not as fast as that of animals. Therefore, chloroplast DNA in plant cells is considered to be DNA barcodes for classifying species. In addition, it is possible to combine with nuclear DNA in order to increase identifiable ability (Kress *et al*., 2005). Almost all DNA barcodes utilized for classification in plants belong Internal Transcribed Spacers (ITS) regions in nuclear genome, and *trn*H-*psb*A (Kress *et al*., 2005), *mat*K, *rbc*L (CBOL Plant Working Group, 2009), *rpo*C1, *rpo*B (Chase *et al*., 2007) regions in chloroplast genome. DNA molecular marker and DNA barcode techniques have been applied to study genetic relationship and classification of *Cordyceps* genus. Kuo *et al*. (2005) had built a phylogenetic tree of 17 species of *Cordyceps* genus based on ITS1-18S rRNA-ITS2 region. In 2007, Sung's study demonstrated that molecular markers *nrSSU*, *nrLSU*, *tef1*, *tub*, *atp6*, *rpb1* and *rpb2* work well in identifying 162 different strains of this genus (Sung *et al*., 2007).

In this study, seven *Cordyceps* samples were investigated based on the analysis of ITS1-5.8S-ITS2 region. The obtained results would contribute an additional DNA database for specifically identifying species in *Cordyceps* genus in Vietnam.

MATERIALS AND METHODS

Materials

Seven fresh fungal strains of *Cordyceps* genus were provided by Agricutural Genetics Institute, Ministry of Agriculture and Rural Development (C1, C2), market (C3), Institute for Regional Research and Development, Ministry of Science and Technology (C4, C5, C6); THD Company (C7) (Figure 1).

Methods

Total DNA extraction and purification

Total DNA of seven of *Cordyceps* specimens were extracted following CTAB method described by Doyle and Doyle (1990). Briefly, 50 mg of each sample was well-grounded in liquid nitrogen. Cell wall and membrane were degraded by suspending in 65^{o}C buffer composed of 1.4 M NaCl (Scharlau, Spain), 0.1 M Tris - HCl (Biobasic, Canada) pH 8.0, 20 mM EDTA (Merck, Germany) pH 8.0, 2% CTAB (Affymetrix, USA), 1% PVP (Sigma-Aldrich, USA), and 1% β-mercaptoethanol (Sigma-Aldrich, USA) for 60 min. The mixture was then hold at room temperature for 5 min to cool down. Cell fragments were removed by adding one volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 12000 rpm for 20 min at 4^{o}C. The supernatants were transferred to new

tubes, added 1 µL RNAse (Thermo Fisher Scientific, USA) and incubated for 15 min at 37°C. The supernatants were purified by adding cloroform:isoamy alcohol (24:1, v:v), centrifuging at 12000 rpm for 20 min at 4°C, transferring to clean tubes. DNA was then precipitated by two volume of absolute ethanol and one tenth of 0.3 M CH_3COONa for 20 min at 4°C, centrifuged at 12000 rpm for 15 min at 4°C. The precipitated DNA was washed with 70% ethanol and air-dried. Finally, the DNA was resuspended in sterilized deionized water for further uses.

Figure 1. Selected samples used for molecular study. Samples C1, C2, C3, C5, C6, C7 were marked as number 1, 2, 3, 5, 6, 7

Amplification of ITS1-5.8S-ITS2 region and PCR product purification

The target DNA region was amplified using primers designed based on the GenBank sequences as following 5'-TCCGTAGGTGAACCTGCGGT-3' (Cor-ITS1-F) and 5'-TCCTCCGCTTAT TGATATGC-3' (Cor-ITS4-R). The reaction was carried out in a volume of 20 µL containing 1X DreamTaq buffer, 200 mM of each dNTP, 2.5 µM of each primer, 1.5 mM $MgCl_2$, 0.75 units of Dream Taq DNA polymerase (Thermo Fisher Scientific, USA) and 50 ng of template DNA. The PCR was performed by IBM Veriti (Applied Biosystems, USA) for 2 min at 95°C denaturation, 35 amplification cycles (30 s at 95°C denaturation, 30 s at 56°C annealing, and 1 min at 72°C extension), 5 min at 72°C extension, then hold at 4°C. The amplified products were purified using GeneJET™ PCR Purification Kit (ThermoFisher Scientific, USA) as described by the manufacturer, then screened on 0.8% agarose gel.

Sequence analysis and alignment

Purified DNA was sequenced by ABI 3500 Genetic Analyzer applying BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). The sequencing mixture was prepared in total volume of 15 µL containing 1X BigDye buffer, BigDye, 3.2 pM primer, and 200 ng purified DNA. The PCR was performed by IBM Veriti (Applied Biosystems, USA) for 1 min at 96°C denaturation, 25 amplification cycles (10 s at 96°C, 5 s at 50°C,

and 4 min at 60°C), then hold at 4°C. After amplification, PCR product was purified by ethanol/EDTA method. Briefly, 5 µL of 125 mM EDTA and 60 µL of absolute ethanol were added to the tube of PCR product and kept at room temperature for 15 min. The supernatant was discarded after centrifuging at 12000 rpm for 15 min. Next, 60 µL of 70% ethanol was added and centrifuged again at 10000 rpm for 10 min. The acquired pellet contained precipitated DNA was dried. The purified DNA was then denatured by adding 10 µL of Hi-Di Formamide at 95^{o}C for 5 min. The samples were loaded to each well of a sample tray, and performed electrophoresis in 80 cm × 50 µm capillary tubes with POP-4 polymer (ABI, USA). The nucleotide sequence of each sample was identified both forward and reverse directions.

All raw obtained sequences were analysed using Bioedit 7.2.6 softwares (Hall, 1999). Pairwise distance was performed using MEGA 7.0 (Kumar *et al.*, 2016). The phylogenetic trees were constructed using Maximum Likelihood methods with bootstrap value equal 1000.

RESULTS

Efficiency of DNA extraction and PCR amplification of target region

Total DNA of all samples were extracted by CTAB method. The obtained genomic DNA in each lane performed an explicit band without any smear in agarose gel (Figure 2A). Additionally, the total amount DNA of these specimens ranged from 6.8 to 7.4 µg, and the purity of extracted DNA was excellent as evident by A260/A280 ratio ranging from 1.72 to 1.97 and A260/A230 ratio was >2. They all indicate that obtained genomic DNA of samples meet quality and quantity requirements for molecular analyses.

Amplicons acquired with specific primers for ITS1-5.8S-ITS2 region represented precise length as expected (650 bp). The success rates of PCR amplification were high for all samples (Figure 2B).

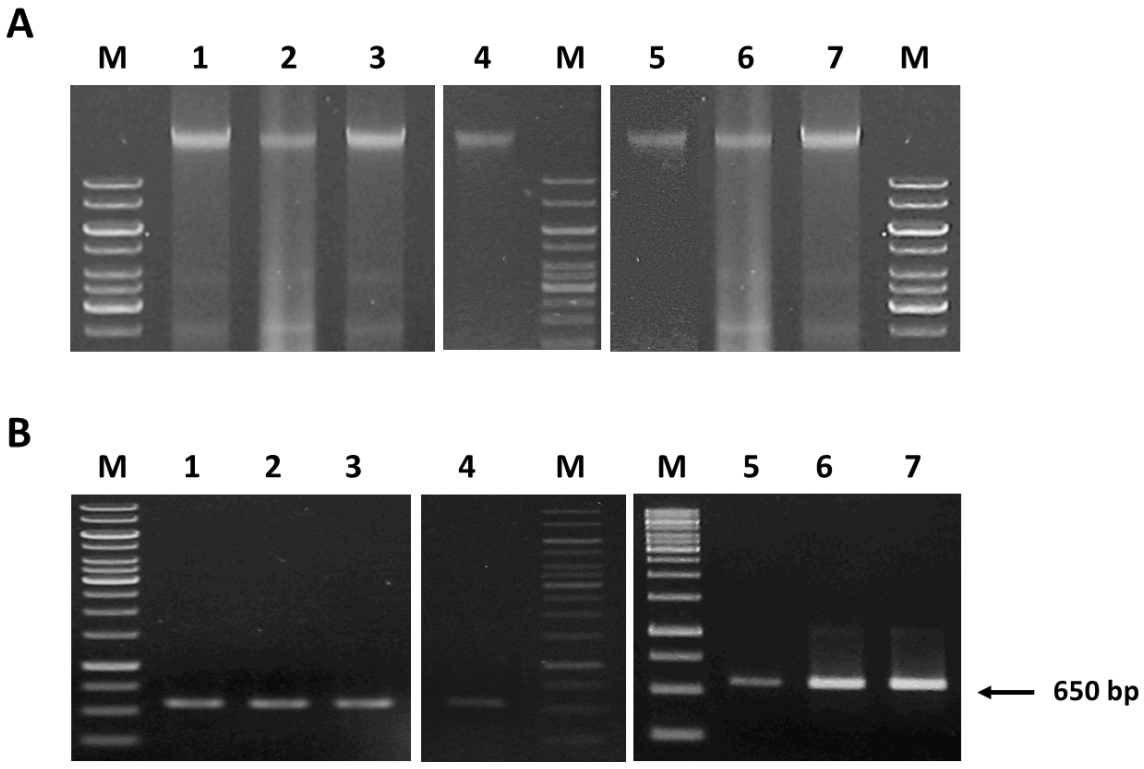

Figure 2. Electrophoresis of genomic DNA extraction (A) and PCR products of amplified target region (B).

Assessment of barcoding gap and authentication ability

To estimate the identification ability using DNA barcoding, BLAST tool was used for determining the identity of a sample based on the best hit of the query sequence, and the E-value for the match must be less than the cut-off value. Accordingly, sequences of typical representatives of *Cordyceps* genus were retrieved to construct a reference sequence library for alignment with obtained sequences in this study.

The results showed that the target DNA region was successfully sequenced. In this analysis, the sequences of six samples C1, C2, C3, C5, C6, C7 were completely similar to each other, while there was a pretty difference from C4 specimen. Comparison of the target region between reference sequences and examined ones revealed that the variability of ITS1 and ITS2 regions is higher than that of 5.8S region. Using three ITS1-5.8S-ITS2 sequences of C1-C3 samples as a query yielded the best matches which were 99.82% identical to ITS1-5.8S-ITS2 sequences of known species *C. militaris*. These results indicated that the samples C1-C3, C5-C7 were most closely related to *C. militaris*. Likewise, alignment of the ITS1-5.8S-ITS2 sequence of C4 exhibited a high degree of homology (99.81%) to *C. sinensis*, which could be implied that they had the close phylogenetic relationship (Table 1).

Table 1. Average similarity of ITS1-5.8S-ITS2 region.

Reference species	Average similarity (%)	
	C1-C3, C5-C7	C4
Cordyceps brongniartii	91.53	82.47
Cordyceps cardinalis	86.30	81.88
Cordyceps cicadae	88.69	86.35
Cordyceps confragosa	90.07	83.00
Cordyceps cylindrica	85.93	88.07
Cordyceps emeiensis	85.85	85.46
Cordyceps gunnii	82.86	89.56
Cordyceps militaris	**99.82**	84.41
Cordyceps morakotii	88.45	86.16
Cordyceps ninchukispora	89.56	87.97
Cordyceps ningxiaensis	95.93	84.39
Cordyceps ochraceostromata	88.43	87.21
Cordyceps pleuricapitata	83.25	83.49
Cordyceps pruinosa	89.62	87.10
Cordyceps pseudomilitaris	87.99	83.61
Cordyceps scarabaeicola	91.68	82.99
Cordyceps scarabaeucika	91.78	82.50
Cordyceps spegazzinii	88.95	82.56
Cordyceps sinensis	84.24	**99.81**
Cordyceps takaomontana	88.43	86.29

Phylogenetic reconstruction

Sequence analyses of the ITS1-5.8S-ITS2 region were used for reconstructing phylogenetic tree based on Maximum Likelihood method. Twenty selected reference sequences belonged to the *Cordyceps* genus served as representative and *Akanthomyces cinereus* was an outgroup. Corresponding to the phylogeny analysis, samples C1-C3, C5-C7 obviously revealed the closest relationships to *C. militaris*. A relevant bootstrap value at 99 by Maximum Likelihood method indicated that a confidence interval was eligible for genetic correlation of these species. For the sample C4, *C. sinensis* was inferred to be the nearest neighbors with the average similarities at 99.81%. This evaluation was confirmed evidently by the result presented in the phylogenetic tree with extremely high bootstrap value (Figure 3).

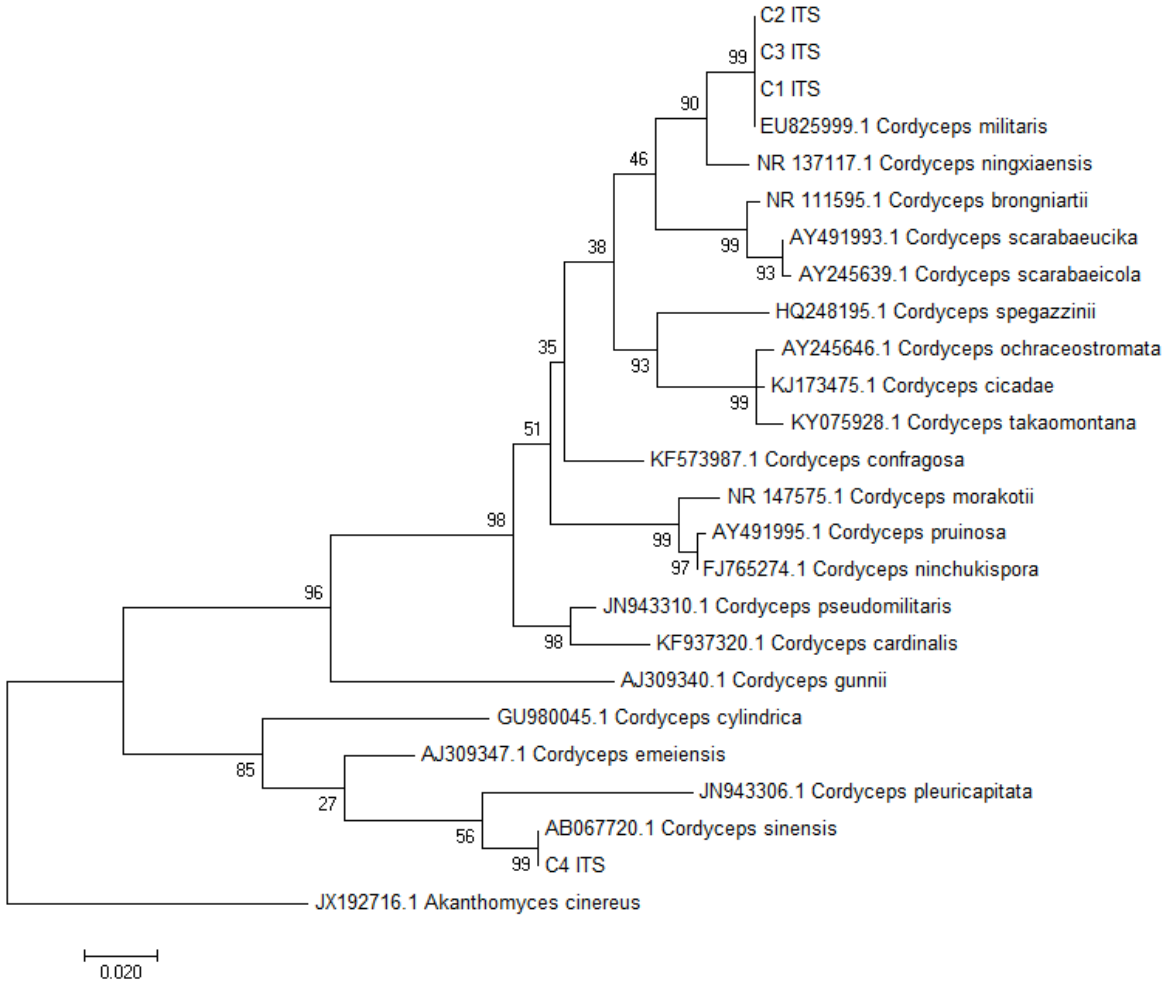

Figure 3. Phylogenetic tree based on ITS1-5.8S-ITS2 of the *Cordyceps* genus imaged by Maximum Likelihood methods. Bootstrap values are indicated below the node.

DISCUSSION

A number of studies which aim to find suitable DNA markers for species identification have been conducted over many decades. Different regions as well as combination thereof have been examined and proposed as preferred plant barcodes. In this study, selected species of *Cordyceps* genus could be identified using the ITS1-5.8S-ITS2 region. The findings on ITS regions are congruent with similar previous study (Kuo *et al.*, 2005). Due to quick evolution and considerable variation, ITS sequences are suitable for utilizing as standard DNA barcodes for taxonomy classification at both the infrageneric and intergeneric levels (Liu *et al.*, 2009; Tripathi *et al.*, 2013; Keskin *et al.*, 2017). Based on ITS1- 18S rRNA- ITS2 region, 17 distinct strains belonged to the *Cordyceps* genus were classified into 5 major group and *C. sinensis* speciments generated a seperate group (Kuo *et al.*, 2005). In 2007, Sung *et al.* built phylogeny of 162 different species of *Cordyceps* genus by using 5 to 7 DNA markers including *nrSSU* (nuclear ribosomal small subunits), *nrLSU* (nuclear ribosomal large subunits), *tef1* (the elongation factor 1α), *tub* (β-tubulin), *atp6* (mitochondrial ATP), *rpb1* and *rpb2* (the largest and the second largest subunits of RNA polymerase II) and suggested that the discrimination at species level need to combine multiple DNA barcodes and morphology characteristics (Sung *et al.*, 2007).

In the genus of *Cordyceps*, *C. militaris* and especially *C. sinensis* are highly commercially valuable species, therefore, it is special important to control and accurately determine these species in trade transactions. In the current study, samples were discriminated pronouncedly based on a combination of morphology of plants and average similarities' assessment between query and reference sequences. The results indicated the ITS1-5.8S-ITS2 region was efficient in distinguishing *C. militaris* and *C. sinensis* species that contributes additional reference sequences for *Cordyceps* species in Vietnam as well as confirms molecular discrimination ability of ITS regions.

CONCLUSION

The results obtained in this study indicated that similarity and phylogenetic analysis based on ITS1-5.8S-ITS2 sequence is the potential DNA barcode to discriminate species of *Cordyceps* genus. These findings can support morphological classification method and generate a foundation for conservation and commercialization of the important traditional medicines in Vietnam.

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