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CLASSIFICATION OF SOME COMMERCIAL LINGZHI (*Ganoderma* spp.) ACCESSIONS IN VIETNAM BY ITS-BASED DNA BARCODE

Phân loại một số mẫu lingzhi thương mại (*Ganoderma* spp.) tại Việt Nam theo mã vạch DNA dựa trên vùng đệm sao chép nội

Ho Viet The*, Vo Thi Ngoc Ha, Le Ngoc Giau

Ho Chi Minh City University of Food Industry, 140 Le Trong Tan St., Tan Phu District, Ho Chi Minh City, Vietnam

* Corresponding author: Ho Viet The (E-mail: thehv@hufi.edu.vn)
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Abstract. In Vietnam, lingzhi is mainly used for medicinal purposes with a high market value leading to the increasing production in recent years. However, the identification and classification of this fungus are inaccurate due mainly to morphological characteristics. In this study, the ITS-based DNA barcode was used to analyze the genetic composition of 10 commercial lingzhi genotypes collected from different regions across the country. The results show that there exists genetic diversity of 10 lingzhi samples. Based on Kimura 2-parameter model, the genetic distances among studied accessions range from 0.000 to 0.047. Seven of ten accessions were reclassified as *G. lingzhi* and three accessions were identified as *G. lucidum*. In addition, 19 nucleotide sites differed among fungal samples were also reported. There is a high potential of using ITS-based DNA barcode for the identification and classification as well as providing information on the genetic relationship between lingzhi varieties.

Keywords*:* DNA barcode, diversity, *Ganoderma* spp., ITS, molecular markers

T**óm tắt.** Tại Việt Nam, nấm linh chi chủ yếu được sử dụng để làm thuốc. Loại nấm này có giá trị thị trường cao nên sản lượng tăng nhanh trong những năm gần đây. Tuy nhiên, việc xác định và phân loại loại nấm linh chi chủ yếu dựa vào đặc điểm hình thái dẫn tới độ chính xác chưa cao. Trong nghiên cứu này, mã vạch DNA dựa trên vùng đệm sao chép nội (ITS-based DNA barcode) được sử dụng để phân tích cấu trúc di truyền của 10 mẫu nấm linh chi thương mại được thu thập từ các khu vực khác nhau trên cả nước. Kết quả cho thấy có sự đa dạng di truyền của 10 mẫu nấm linh chi. Dựa vào phân tích theo mô hình Kimura 2- parameter, khoảng cách di truyền giữa các mẫu nấm dao động từ 0,000 đến 0,047. Bảy trong số mười mẫu nấm được xác định thuộc loài *G. lingzhi* và ba mẫu còn lại được xác định thuộc loài *G. lucidum*. Ngoài ra, nghiên cứu này cũng tìm ra được 19 vị trí nucleotide khác nhau giữa các mẫu nấm. Mã vạch DNA dựa trên vùng đệm sao chép nội có triển vọng lớn trong việc nhận dạng và phân loại cũng như cung cấp thông tin về mối quan hệ di truyền giữa các giống nấm linh chi.

Từ khóa: chỉ thị phân tử, đa dạng, *Ganoderma* spp., mã vạch DNA

1 Introduction

Lingzhi (*Ganoderma* spp.) is a medical mushroom commonly used in Asian countries and parts of the world. This mushroom possesses several medical properties such as anti-tumor, anti-inflammatory, antiviral (anti-HIV), and antibacterial activity, and it can lower blood pressure, cholesterol, and blood sugar levels [1]. Despite the medical importance and high market value, the taxonomic studies of this mushroom are insufficient. Presently, the morphological characteristic is mostly applied for lingzhi classification due to its easiness to perform and carry out on the field at a low cost. However, there are several limitations of this method such as low number, complex inheritance pattern, and vulnerable to changes in the environment [2]. The confused identification makes it more difficult for authenticating cultivar correctly and leads to variation in controlling lingzhi quality.

To overcome the disadvantages in morphology-based taxonomy, molecular markers have been used for identifying the genetic relationships among mushrooms because of fast, specific, reliable, and sensitive features [3, 4]. Among newly developed molecular markers, the DNA barcode is considered as the standardized marker to distinguish among species and has been used intensively for identifying at the species level. At the Fourth International Barcode of Life Conference, Internal Transcribed Spacer (ITS) region has been proposed as a standard region for fungi identification because the ITS region is highly conserved among interspecies but variable between interspecies and is very easy to amplify even from small quantities of DNA. Thus, the ITS-based DNA barcode has been intensively utilized in the identification and evaluation of the genetic diversity of lingzhi. For example, Zheng performing the genetic analysis of 37 *Ganoderma* spp. genotypes in China showed high levels of genetic diversity [5]. Then in 2012, Park et al. analyzed the genetic diversity of 59 *Ganoderma* spp. accessions in Korea [6]. In 2015, Pawlik et al. studied the genetic diversity of 14 *G. lucidum* strains from different geographical regions using the ITS region sequence. From the sequences obtained, the genetic relationships between the strains analyzed were determined [1]. Other studies also reported that ITS shows higher performance compared with other plastid markers [7, 8, 9, 10].

Up to 76 species belonging to the *Ganoderma* genus have been identified and approximately 20 of them are used for medical purposes [8]. Most of the lingzhi genotypes in Vietnam are traditionally considered as *G. lucicum* [11, 12, 13]. However, recent studies have shown that lingzhi distributed in East Asia belongs to the *G. lingzhi* species [14], and there is a difference in the chemical composition of these two fungus species [7]. The study on genetic diversity and molecular identification of lingzhi in Vietnam has not attracted enough attention. To our best knowledge, only Le and Tran studied the biological characteristics and productivity of lingzhi using random amplified polymorphic DNA marker [11]. In this study, the genetic diversity of 10 lingzhi accessions collected nationwide were analyzed using ITS-based DNA barcode markers. The obtained results could provide scientific information for the classification, identification, and authentication of lingzhi in Vietnam.

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2 Materials and methods

2.1 Materials

Lingzhi samples were collected from research institutes, universities, and companies in different parts of Vietnam (Figure 1 and Table 1). After collecting, the samples were grown on the solid PDA (potato, glucose, and agar) medium and targeted for DNA extraction.

Fig. 1. Targeted areas for collecting lingzhi samples

Table 1. Lingzhi samples collected for genetic characterization

No.	Collected location	Sample code
1	Tien Giang Applied Science and Technology Research Center	TG1
2	Tien Giang Applied Science and Technology Research Center	TG2
3	Lam Dong Center for Science and Technology Application	LD
4	Healthy Fungi Company Limited, Da Nang	DN
5	Hamlet 5, Binh My commune, Cu Chi district, Ho Chi Minh City	CC
6	Dinh Hoa Ward, Thu Dau Mot city, Binh Duong	BD1
7	Experimental Research Center of Thu Dau Mot University	BD2
8	Tan Hung village, Tan Thoi commune, Tan Phu Dong district, Tien Giang	TG3
9	83 Tran Quy cap, Tu An Ward, Buon Me Thuoc City, Dak Lak	DLk
10	Center for Mushroom Research and Development, Tu Liem District, Hanoi	HN

2.2 Methods

DNA extraction

Total DNA was extracted from lingzhi mycelia using the method described by Porebski et al. [15]. The DNA quality was then tested using electrophoresis on 1% agarose gel in Tris-Acetate-EDTA 1X buffer and stained with Gelred dye (Biotium, USA). The samples were observed under ultraviolet light using Quantum-ST4 3000 gel reader (Montreal–Biotech, Canada). DNA concentrations were determined using a spectrophotometer (Optima SP 3000 nano UV-VIS, Japan).

ITS amplification

The ITS region was amplified using the composition of PCR reactions as follows: 7.5 µL 2X Mytaq Red Mix (Bioline, UK), 20 ng DNA, 0.2 µM primer (ITS1_FW 5′ TCCGTAGGTGAACCTGCGG 3′ and ITS2_RV 5′ TCCTCCGCTTATTGATATGC 3′) [16], and PCR water for a final volume of 15 µl. The PCR reaction conditions are as follows: initial denaturation at 95 °C for 2 minutes; then 35 cycles of 30 seconds at 95 °C, 30 seconds at 55 °C, 1 minute at 72 °C, and finally five minutes at 72 °C to complete the reaction. All reactions were carried out in a SureCycler 8800 Thermal Cycler (Agilent, USA). The PCR products were electrophoresed using 1% agarose gel to check the presence or absence of bands. PCR amplification products were then purified using ISOLATE II PCR and Gel Kit (Bioline, UK) and then sequenced using the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystem, USA). The products were next run on an ABI 3100 DNA analyzer (Applied Biosystem, USA). The obtained sequences were submitted to GenBank and made publicly accessible under the accession numbers listed in Table 2.

Data analysis

The obtained electropherograms were edited using FinchTV (Digital World Biology Products, USA). The sequences were blasted on NCBI BLAST under program BLASTN (National Center for Biotechnology Information, USA). All of the sequences were aligned using the MUSCLE method. Phylogenetic analysis was conducted according to the neighbor-joining (NJ); pairwise deletion was used for gaps/missing data on MEGA 6.0 with 1000 bootstrap replicates. Bootstrap support (BS) was categorized as strong (>85%), moderate (70–85%), weak (50–69%), and poor (<50%) [17].

3 Results and discussion

3.1 PCR and DNA sequence

There is a concern about the unreliability of PCR in the ITS region in different organisms. An ITS marker was identified as the least PCR success with only three of twelve invasive grass species in Australia [18]. However, in our study, the used PCR protocol is reliable for the ITS region with complete PCR reactions (Figure 2). Similarly, a high success rate of PCR in the ITS gene was reported by different research groups. This is also consistent with Chen et al. when they claimed that ITS is the most suitable region for

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DNA barcoding in medical plants after surveying seven candidates with DNA barcodes namely *psbA-trnH, matK, rbcL, rpoC1, ycf5*, ITS2, and ITS [19].

The length of ITS sequences is 584 bp on average, ranging from 536 bp to 615 bp. The obtained sequences were then submitted to GenBank and shown in Table 2. Basic Local Alignment Tool (BLAST) was used to compare the sequence homology of the amplified sequences and sequences from GenBank. The BLAST similarity ranges from 98.76 to 99.83%. The sequence homology of lingzhi accessions in this study is shown in Table 2. Three of ten lingzhi accessions were identified as *G. lucidum*, whereas the remaining are *G. lingzhi*. Previous studies have also shown the incorrect report of lingzhi as *G. lucidum* worldwide such as Africa, Oceania, America, Asia, and Europe [20]. Other results from Kwon also showed that lingzhi cultivated in Korea is *G. lingzhi*, not *G. lucidum*. In the same year, Liao et al. also indicated that lingzhi from East Asia was different from those from Europe, and lingzhi could be misnamed in Asia [8].

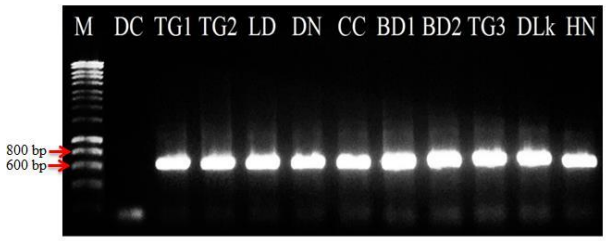

Fig. 2. Agarose gel electrophoresis of PCR amplified products of ITS regions of *Ganoderma* spp. accessions. (M: HyperLadder™ (Bioline, UK), DC: negative control; the following lanes are corresponding to sample codes indicated in Table 1)

Table 2. Lingzhi samples collected for genetic characterization and corresponding Genbank accession number

Accessions	Genbank accession number	Sequence length (bp)	Targeting species	BLAST similarity (%)	Sequence cover (%)	E-value
TG1	MN372058	605	*G. lingzhi*	99.67	99	0.0
TG2	MN372059	602	*G. lingzhi*	99.66	99	0.0
LD	MN372060	567	*G. lingzhi*	98.76	99	0.0
DN	MN372061	581	*G. lingzhi*	98.96	99	0.0
CC	MN372062	584	*G. lingzhi*	98.83	100	0.0
BD1	MN372063	536	*G. lucidum*	98.88	100	0.0
BD2	MN372064	587	*G. lucidum*	99.49	100	0.0
TG3	MN372065	586	*G. lucidum*	99.83	100	0.0
DLk	MN372066	586	*G. lingzhi*	99.83	99	0.0
HN	MN372067	615	*G. lingzhi*	99.50	98	0.0

The alignment of DNA sequences of the 10 lingzhi samples is shown in Table 3. Among 19 variable sites, there were unique sites found specifically to be *G. lucidum*. These unique nucleotide sites can be used as a diagnostic character for discriminating different lingzhi genotypes. Such characters have been widely applied in molecular identification studies of different plant species such as *Taxus* L. [12], onion [6], and *Terminalia* sp. [15]. By comparing several DNA barcode markers, namely *rbcL*, *matK*, ITS, and *psbA-trnH*, Mishra et al. reported that the ITS marker has the highest efficiency for identifying *Decalepsis* at the species level [15]. The classification of lingzhi genotypes could be important because a previous study has shown that there is a difference in the secondary metabolites between *G. lucidum* and *G. lingzhi* where the former contains much fewer triterpenic acids than the latter. This feature leads to the bitter taste of *G. lingzhi* [5].

3.2 Phylogenetic trees

The genetic distance among lingzhi accessions based on the Kimura-2 parameter (K2P) is shown in Table 4. The lowest distance is 0.000, while the highest is 0.047. The low distance shows the close genetic relationship among these accessions. Whereas, the sample collected from Daklak province (DLk) is more distant from the samples collected from Lam Dong (LD) and Dong Nai (DN) although all accessions are in a narrow geographic area.

Table 3. Variable sites in 10 lingzhi accessions based on ITS-based DNA barcode

Accession	Position																		
	104	106	107	109	110	112	113	117	138	140	176	200	381	430	432	483	502	543	554
TG1	T	C	G	G	G	A	C	T	A	A	A	A	A	A	C	C	C	T	G
TG2	T	C	G	G	G	A	C	T	A	A	A	A	A	A	C	C	C	T	G
LD	T	C	G	G	G	A	C	T	A	A	A	A	A	A	C	C	C	T	G
DN	T	C	G	G	G	A	C	T	A	A	A	A	A	A	C	C	C	T	G
CC	T	C	G	G	G	A	C	T	A	A	A	A	A	A	C	C	C	T	G
BD1	C	T	A	A	A	G	G	C	G	G	T	G	G	G	T	G	T	G	A
BD2	C	T	A	A	A	G	G	C	G	G	T	G	G	G	T	G	T	G	A
TG3	C	T	A	A	A	G	G	C	G	G	T	G	G	G	T	G	T	G	A
DLk	T	C	G	G	G	A	C	T	A	A	A	A	A	A	C	C	C	T	G
HN	T	C	G	G	G	A	C	T	A	A	A	A	A	A	C	C	C	T	G

Table 4. Genetic distance among 10 lingzhi accession based on K2P method

	TG1	TG2	LD	DN	CC	BD1	BD2	TG3	DLk	HN
TG1										
TG2	0.000									
LD	0.000	0.000								
DN	0.002	0.002	0.002							

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	TG1	TG2	LD	DN	CC	BD1	BD2	TG3	DLk	HN
CC	0.000	0.000	0.000	0.002						
BD1	0.040	0.040	0.040	0.043	0.040					
BD2	0.040	0.040	0.040	0.043	0.040	0.000				
TG3	0.002	0.002	0.002	0.004	0.002	0.043	0.043			
DLk	0.045	0.045	0.045	0.047	0.045	0.004	0.004	0.043		
HN	0.000	0.000	0.000	0.002	0.000	0.040	0.040	0.002	0.045	

The phylogenetic tree constructed by using MEGA 6.0 with the neighbor-joining (NJ) method is shown in Figure 3. The studied lingzhi accessions were divided into two main groups on the basis of NJ analysis in Figure 3. Apparently, three accessions that are identified as *G. lucidum* consisting of TG3, BD1, and BD2 are clustered in a separated group, whereas the remaining seven accessions that are identified as *G. lingzhi* consisting of TG1, TG2, LD, DN, CC, DLk, and HN are put together. Generally, the studied accessions are not distributed in groups corresponding to geographical locations. Some accessions are grouped together, but they are collected from distant locations such as HN from the North (Hanoi), LD and DLk from the Central region (Lam Dong and Daklak, respectively), TG, DN, and CC from the Southern region (Tien Giang, Dong Nai, Ho Chi Minh City, respectively). This result suggests there is a mixture of accessions.

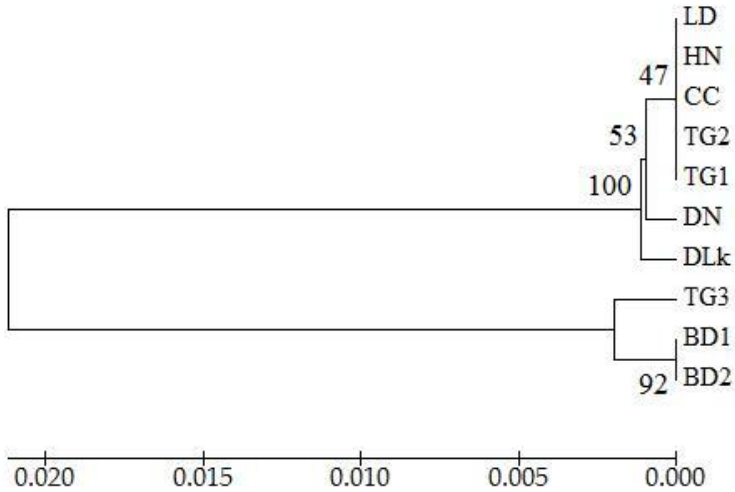

Fig. 3. Phylogenetic tree based on ITS region sequences for the 10 lingzhi accessions. The evolutionary distances were computed using the Kimura 2-parameter method. The scale bar showing the units of the number of base substitutions per site. The number on the branch indicates bootstrap value.

4 Conclusions

DNA barcoding has been widely chosen for authenticating materials from herbal plants, product substitution, and contamination. Our results show that the ITS marker is effective to classify lingzhi accessions. The advantages of DNA barcoding is that it could make the identification of mushroom species easier for non-experts in molecular biology because time and labor could be reduced significantly compared with morphological identification. The obtained results in this study prove that the use of ITS-based DNA barcodes is successful for amplification, identification, and discrimination at the species level of lingzhi.

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