GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Phylogenetic analysis of widely cultivated *Ganoderma* in China based on the mitochondrial V4-V6 region of SSU rDNA

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Genet. Mol. Res. 14 (1): 886-897 (2015)
Received February 6, 2014
Accepted May 15, 2014
Published February 2, 2015
DOI http://dx.doi.org/10.4238/2015.February.2.12

ABSTRACT. *Ganoderma* mushroom is one of the most prescribed traditional medicines and has been used for centuries, particularly in China, Japan, Korea, and other Asian countries. In this study, different strains of *Ganoderma* spp and the genetic relationships of the closely related strains were identified and investigated based on the V4-V6 region of mitochondrial small subunit ribosomal DNA of the *Ganoderma* species. The sizes of the mitochondrial ribosomal DNA regions from different *Ganoderma* species showed 2 types of sequences, 2.0 or 0.5 kb. A phylogenetic tree was constructed, which revealed a high level of genetic diversity in *Ganoderma* species. *Ganoderma lucidum* G05 and *G. eupense* G09 strains were clustered into a *G. resinaceum* group. *Ganoderma* spp G29 and G22 strains were clustered into a *G. lucidum* group. However, *Ganoderma* spp G19, G20, and G21 strains were

clustered into a single group, the *G. lucidum* AF214475, *G. sinense*, *G. strum* G17, *G. strum* G36, and *G. sinense* G10 strains contained an intron and were clustered into other groups.

Key words: *Ganoderma* spp; *Ganoderma* identification and classification; Mitochondrial small subunit rDNA; Phylogenetic analysis.

INTRODUCTION

Ganoderma lucidum (Lingzhi or Reishi) is a bitter mushroom with remarkable health benefits (Batra et al., 2013). It has long been prescribed to prevent and treat various human diseases, particularly in China, Japan, and Korea (Boh, 2013). Various bioactive substances have been isolated and identified from *G. lucidum*, such as triterpenoids (Wu et al., 2013), polysaccharides (Xu et al., 2011), and fungal immunomodulatory proteins (Li et al., 2011). *G. lucidum* was described by Curtis based on material from England and the epithet was sanctioned by Fries (Wang et al., 2012). Within the genus of *Ganoderma*, more than 250 taxonomic names have been reported worldwide (Moncalvo et al., 1994), with 98 genera distributed throughout China. However, only 2 species, *G. lucidum* (Leyss. Ex Fr.) and *G. sinense* Zhao, Xu et Zhang, were described in the pharmacopoeia of China in 2005. One species, *G. tsuage* Murr, was regarded for its use in health products (Zhou et al., 2007b). Furthermore, *G. lucidum*, design as Lingzhi mushroom (Chinese), is also listed in the American Herbal Pharmacopoeia and Therapeutic Compendium (Sanodiya et al., 2009). Although *G. lucidum* has been widely considered to be a Chinese species of Ling-zhi, there are approximately 51 species with similar morphological characteristics (Li et al., 2013). Traditional identification of *Ganoderma* species was based on morphological features (Wu et al., 1994), physiological and developmental characteristics, and chemical components such as secondary metabolites (Hong and Jung, 2004; Xing et al., 2004). The Japanese believed that the true *Ganoderma* is red, but Chinese people believe that the true *Ganoderma* is black. Black *Ganoderma* has a higher medicinal value than the red mushroom (Mayzumi et al., 1997). However, the change of fruiting body color from the same genus can be greatly affected by growth conditions. As a result, the concept of species in this genus has not been well established nor universally accepted (Gottlieb et al., 2000).

DNA markers can be used to identify polymorphisms, as the genetic composition is unique for each species and is not affected by age, physiological conditions, or environmental factors (Chan, 2003). DNA can be extracted from fresh or dried tissue, powders, or mixed drug formulas (Singh et al., 1999; Warude et al., 2003); thus, this procedure is convenient, rapid, and accurate and requires only a small amount of sample. There are various well-established molecular marker techniques that have been widely used for classifying *Ganoderma* strains. These methods include hybridization-based methods (Luo et al., 2005), polymerase chain reaction (PCR)-based methods (Hseu et al., 1996; Luo et al., 2005; Zheng et al., 2007; Wu et al., 2009), and nuclear sequencing-based methods. These sequences contain the nucleotide sequences of a 25S-28S nuclear large subunit ribosomal DNA (rDNA) (Moncalvo et al., 1995a; Lee et al., 2006), 5.8S nuclear small subunit (SSU) rDNA (Latiffah et al., 2002), nuclear internal transcribed spacer (ITS) region rDNA (Moncalvo et al., 1995b; Gottlieb et al., 2000; Smith and Sivasithamparam, 2000; Latiffah et al., 2002), and a specific gene (Zhou et

al., 2008) using nuclear sequencing-based methods. Although phylogenetic studies of nuclear rDNA sequences have provided insights into the relationships among *Ganoderma* species, the relationships between this genus and other genera of the Polyporaceae remain unclear. In contrast, nuclear ITS rDNAs are quite variable and often cannot be aligned accurately between genera and are now commonly used in the systematics of species within a genus (Moncalvo et al., 1995a; Yan et al., 1995). Therefore, the reliability and application of these methods for distinguishing medical *Ganoderma* from other *Ganoderma* species are limited.

The mitochondria of filamentous fungi have uniparental inheritance and their genomes evolve faster than the corresponding nuclear DNA, making them more suitable for differentiating closely related organisms (Ghikas et al., 2010). Mitochondrial DNA is generally considered to be appropriate for phylogenetic analyses at a family level. The small subunit ribosomal DNA (mt SSU rDNAs) were reported to evolve 16 times faster than 18S rDNAs (Bruns and Szaro, 1992), but are less variable than ITS rDNAs. *G. lucidum* is a complex species; thus, distinguishing the subspecies is very difficult. Previous studies have shown that mt SSU rDNA sequences of *Ganoderma* species contained valuable phylogenetic information in conserved domains as well as in variable domains. The variable domains may be useful markers and were helpful for describing phylogenetic groups (Hong et al., 2002; Hong and Jung, 2004).

In this study, we sequenced V4-V6 mt SSU rDNA sequences, which was used to conduct a genetic study of 10 widely cultivated *Ganoderma* strains. The aim of this study was to lay a foundation for establishing a genetic database, which may provide molecular evidence for the selection and breeding of eminent cultivars and to determine genetic relationship in *Ganoderma*.

MATERIAL AND METHODS

Ganoderma materials

Experienced botanists collected plant specimens, including commercial cultivars and wild varieties that represented the great diversification of types from different regions of China as follows: *G. lucidum* GIM 5.251 (G05) from the Microbiological Culture Collection Center of Guangdong Institute of Microbiology; *G. eupense* 51229 (G09) and *G. sinensis* 51332 (G10) from the Agricultural Culture Collection of China (ACCC); black *Ganoderma* spp (G17) and white *Ganoderma* spp (G19) from the Institute of Edible Fungi of Middle China (Wuhan); *Ganoderma* spp (G20) and *G. atrum* (G36) from the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences; *Ganoderma* spp (G22) from the Sichuan Academy of Agricultural Sciences; *Ganoderma* spp (G21) from Shaanxi University of Technology, *Ganoderma* spp (G29) from Shandong Agriculture University. *Ganoderma* mycelia were prepared in liquid culture in our laboratory.

Ganoderma cultures

Stock cultures were maintained in slant tubes at 4°C on improved potato dextrose agar medium, containing 200 g/L potato, 20 g/L dextrose, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 2.5 g/L KH_2PO_4, 10 mg/L vitamin B_1, and 20 g/L agar. Stock cultures were transferred to Petri dishes containing potato dextrose agar and allowed to incubate for 5 days at 28°C. Two agar blocks (φ9 mm)

were obtained and inoculated into a 250-mL Erlenmeyer flask containing 50 mL pre-culture liquid medium, composed of 35 g/L sucrose, 5 g/L peptone, 2.5 g/L yeast extract, 0.5 g/L $MgSO_4$, 1 g/L KH_2PO_4, and 50 mg/L vitamin B_1 at an initial pH of 6.8. The mycelia were maintained on a rotary shaker at 120 rpm and 28°C for 5-10 days and harvested by centrifugation at 5000 *g* for 15 min. After washing 3 times with phosphate-buffered saline, the mycelia were collected by centrifugation and then treated with dry refrigeration to extract genomic DNA.

DNA extraction

DNA extractions were performed using the high-salt and low-pH method (Zhou et al., 2007a). First 1 g material was homogenized in liquid nitrogen in a pre-cooled mortar, transferred to a 50-mL tube containing 5.0 mL pre-warmed (65°C) extraction buffer (1.4% SDS, 100 mM NaAc, pH 4.8, 50 mM EDTA, pH 8.0, 500 mM NaCl, pH 4.8) and mixed gently. The mixture was then placed in a 65°C water bath for 30 min. The homogenate was pelleted by centrifugation at 10,000 *g* for 10 min and the aqueous phase was removed to a new tube containing 2/3 vol. 2.5 M KAc, pH 4.8, solution. After the tube was shaken gently, the sample was incubated for 30 min at 0°C until the protein was precipitated. The protein was pelleted by centrifugation at 10,000 *g* for 10 min at 4°C and the aqueous phase was removed to a new tube containing 2/3 vol. cold isopropanol; the sample was incubated for at least 16 h at -20°C. After centrifugation, the pellet was washed with 70% ethanol, dried, and resuspended in 500 μL TE buffer. The DNA was stored at -20°C.

PCR amplification

Two primers, BMS65 and BMS113, were designed based on the highly conserved V4-V6 sequence of SSU rDNA of *Ganoderma*. The following PCR conditions were used: 10 min at 95°C, 35 cycles (30 s at 95°C, 30 s at 60°C, 2 min at 72°C), and 10 min at 72°C.

Cloning and sequencing

PCR products were purified using the Gel Extraction Mini Kit (Watson Biotechnologies Inc., Taipei, Taiwan), ligated into pMD18-T vectors (Takara, Shiga, Japan), transformed into *Escherichia coli* strain DH5α, and then sequenced (Shanghai Sangon Biological Engineering Technology and Service Co. Ltd., Shanghai, China).

Sequence alignment and analysis

Sequence data were compared to other isolates used and are listed in Table 1. DNA sequences were aligned using the ClustalW program in the biology workbench version 3.2 (Thompson et al., 1994). Final alignments were visually examined and adjusted manually. Phylogenetic analysis was conducted using the MEGA version 2.1 program (Tamura et al., 2011).

Table 1. Sequences of *Ganoderma* used in this study.

Family	Species name	Strain No.	Geographical origin	GenBank accession No.	Reference
Ganodermataceae	*Ganoderma adspersum*	Unknown	UK	DQ661914, DQ661918	NCBI database
	Ganoderma applanatum	CBS 175.30 (IMSNU 32110)	UK	AF248336	Hong and Jung, 2004
		G19	Wuahn, China	No	In the study
	Ganoderma atrum	G17	Wuhan, China	No	In the study
		G36	Shanghai, China	No	In the study
	Ganoderma colossus	CBS 268.88 (IMSNU 32111)	USA	AF248337	Hong and Jung, 2004
	Ganoderma eupense	G09	China	No	In the study
	Ganoderma lobatum	CBS 222.48 (IMSNU 32112)	USA	AF248323, AF248318, AF248316	Hong and Jung, 2004
	Ganoderma lucidum	ATCC 46755 (KCTC 6450)	Canada	AF248328, AF248341, AF214475	Hong et al., 2002
		G05	Guangdong, China	No	In the study
		G20	Shanghai, China	No	In the study
		G21	Shaanxi, China	No	In the study
		G22	Sichuan, China	No	In the study
		G29	Shandong, China	No	In the study
	Ganoderma sinensis	G10	China	No	In the study
	Ganoderma meredithiae	ATCC 64492 (KCTC 6284)	USA	AF248343, AF248344	Hong and Jung, 2004
	Ganoderma oerstedii	ATCC 52411 (KCTC 6286)	Argentina	AF214469	Hong et al., 2002
	Ganoderma oregonense	CBS 177.30 (IMSNU 32116)	Canada	AF214471	Hong et al., 2002
	Ganoderma pfeifferi	CBS 747.84 (KCTC 6512)	Netherlands	AF214478, AF214479	Hong et al., 2002
	Ganoderma resinaceum	CBS 152.27 (IMSNU 32118)	UK	AF214472, AF248334	Hong et al., 2002
	Ganoderma subamboinense	ATCC 52419 (KCTC 6454)	Argentina	AF248348, AF248349	Hong and Jung, 2004
	Ganoderma tsugae	ATCC 46754 (KCTC 6455)	Unknown	AF214473	Hong et al., 2002
Corticiaceae	*Cytidia salicina*	CBS 727.85 (KCTC 6997)	Netherlands	AF214458	Hong et al., 2002
Polyporaceae	*Trametes elegans*	Unknown	China	FJ591058	NCBI database
Hericiaceae	*Hericium coralloides*	IFO 7716 (KCTC 6722)	Japan	AF214462	Hong et al., 2002
Hymenochaetaceae	*Phellinus laevigatus*	CFMR 5640 (IMSNU 30079)	Wisconsin	AF230363	Hong et al., 2002

RESULTS

Sequence analysis of mt SSU rDNA

The full-length sequence of mt SSU rDNA from *G. lucidum* (AF214475) and *G. sinense* (KF673550) was 3506 bp (Li et al., 2013) and contained 1 intron. The sequence of the intron was 1506 bp (1041-2547). However, the full-length mt SSU rDNA sequence from the other *Ganoderma* species was approximately 2000 bp, contained no intron, including *G. applanatum*, *G. lobatum*, and *G. meredithiae* (Hong et al., 2002). The analysis of the full-length sequence revealed that there are 9 variable domains (V1-V9), with an additional intron located between positions V4-V5 (Figure 1).

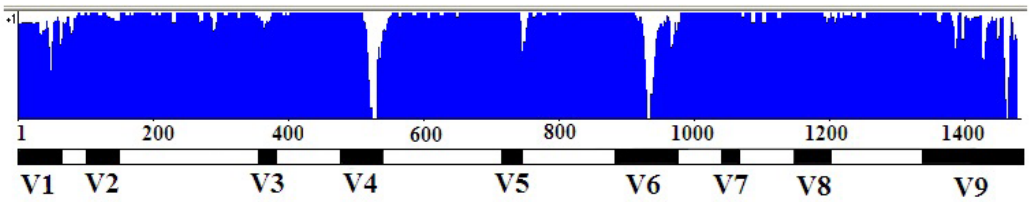

Figure 1. Alignment of 9 variable domains (V1-V9) shown using Vector NTI.

Characteristics of *Ganoderma* mt SSU rDNA

A previous study showed that among the 9 variable domains, V1, V4, V5, V6, and V9 were more variable than the other 4 variable domains (V2, V3, V7, and V8), using 37 mt SSU sequences of *Ganoderma* (Hong and Jung, 2004). Twelve mt SSU sequences of *Ganoderma* were aligned unambiguously, except for V4, V5, and V6 variable domains (Figure 1) that had undergone several insertion/deletion events. The 12 mt SSU full-length sequences of *Ganoderma* represented different species, including *G. adspersum*, *G. applanatum*, *G. colossus*, *G. lobatum*, *G. lucidum*, *G. meredithiae*, *G. oerstedii*, *G. oregonense*, *G. pfeifferi*, *G. resinaceum*, *G. subamboinense*, and *G. tsugae*. Phylogenetic relationships among the 12 *Ganoderma* species were inferred by heuristic search options and compared with the results of the neighbor-joining tree reconstructed by Kimura's two-parameter model. Based on monophyly, branch length, and bootstrap support, *Ganoderma* species were divided into 6 monophyletic groups: I. *G. colossus* group, II. *G. applanatum* group, III. *G. tsugae* group, IV. Asian *G. lucidum* group, V. *G. meredithiae* group, and VI. *G. resinaceum* (Figure 2A). The results are consistent with the results of a previous study (Hong and Jung, 2004). Amplification of full-length sequences of mt SSU sequences is very difficult using PCR, particularly when the gene contains a large intron. Thus, we selected the V4-V6 region of mt SSU rDNA sequences to evaluate the relationships between species. The results were in agreement with full-length sequences (Figure 2B). Thus, the V4-V6 region of mt SSU rDNA sequences can be used as molecular markers for resolving phylogenetic relationships between *Ganoderma* species.

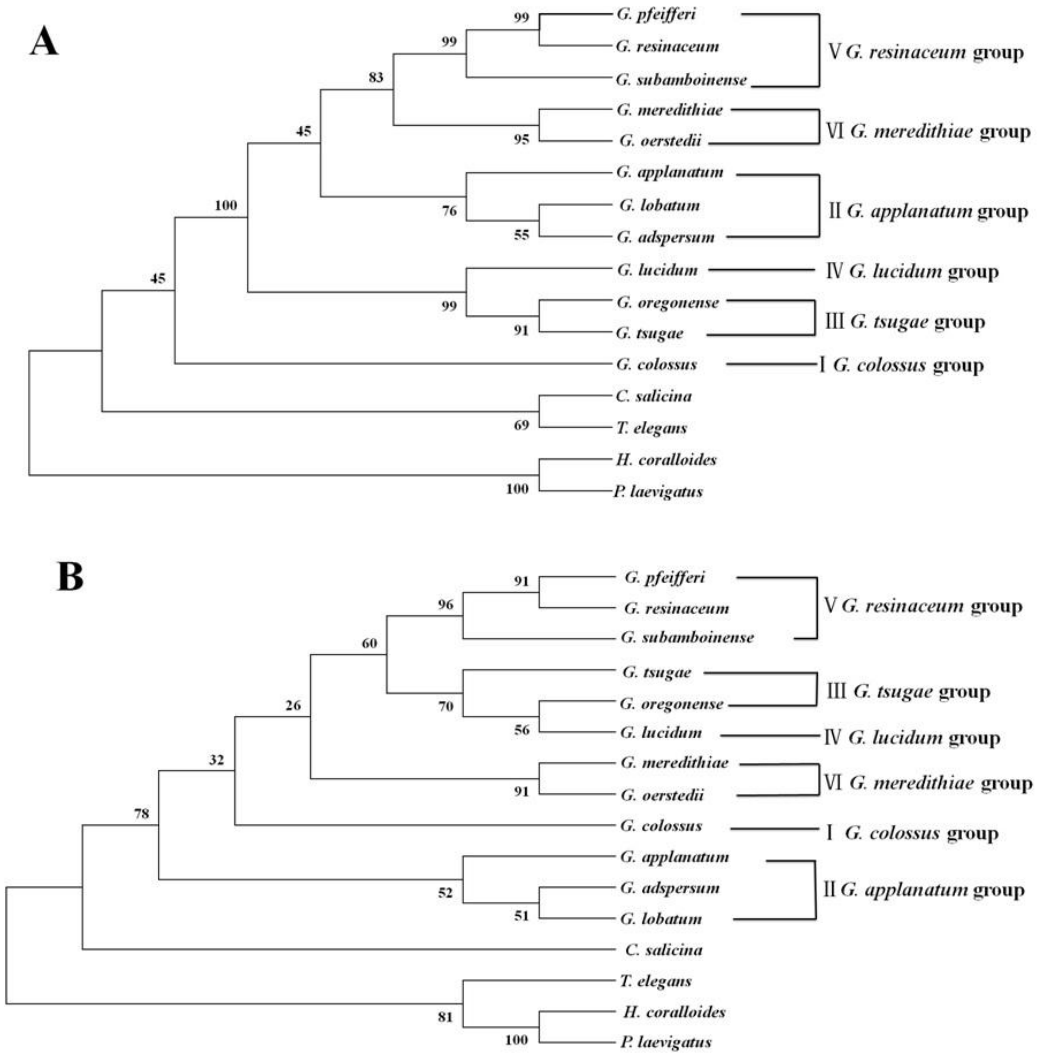

Figure 2. Phylogenetic trees constructed from unambiguously aligned full-length (**A**) or V4-V6 region sequence (**B**) of mt SSU rDNA from *Ganoderma* species as determined by NJ analysis.

Sequence amplification of mt SSU rDNA

The DNA isolated from the mycelia of *Ganoderma* and the primers BMS65 (5'-CTGG TGCCAGAAGACTCGGTAA-3') (512-533) and BMS113 (5'-GACAGCCATGCAACACCT GTA-3') (1043-1063) were used to specifically amplify the V4, V5, and V6 region sequences by PCR. The amplified mt SSU fragments of 7 strains using the primers BMS65 and BMS113 were approximately 550 bp in length for all taxa except *G. sinensis* (G10), *G. atrum* (G17), and *G. atrum* (G36), for which it was approximately 2 kb in length, as visualized on agarose gels stained with ethidium bromide (Figure 3). The 7 strains of PCR products producing bands

of 550 bp included *G. lucidum* (G5), *G. eupense* (G9), *G. applanatum* (G19), and *Ganoderma* spp (G20, G21, G22, G29). Based on nucleotide sequence alignment, the differences in length of the PCR products of *G. sinensis* (G10), *G. atrum* (G17), and *G. atrum* (G36) resulted from a single insert located approximately 1506 bp between positions V4-V5 (Figure 4).

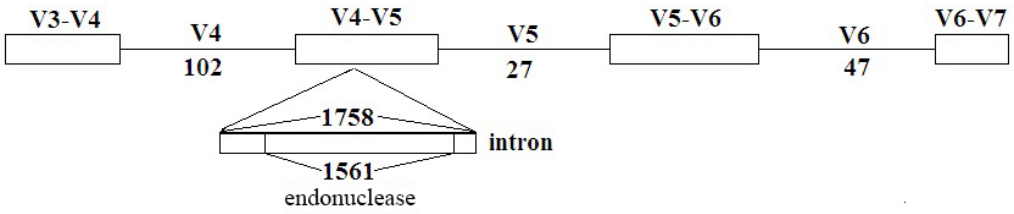

Figure 3. Locations of 3 variable domains (V4-V6) are shown as boxes in the diagram of the mt SSU rDNA gene of *Ganoderma atrum* G36. The position of the group II intron found in 3 strains was indicated between V4 and V5 domains.

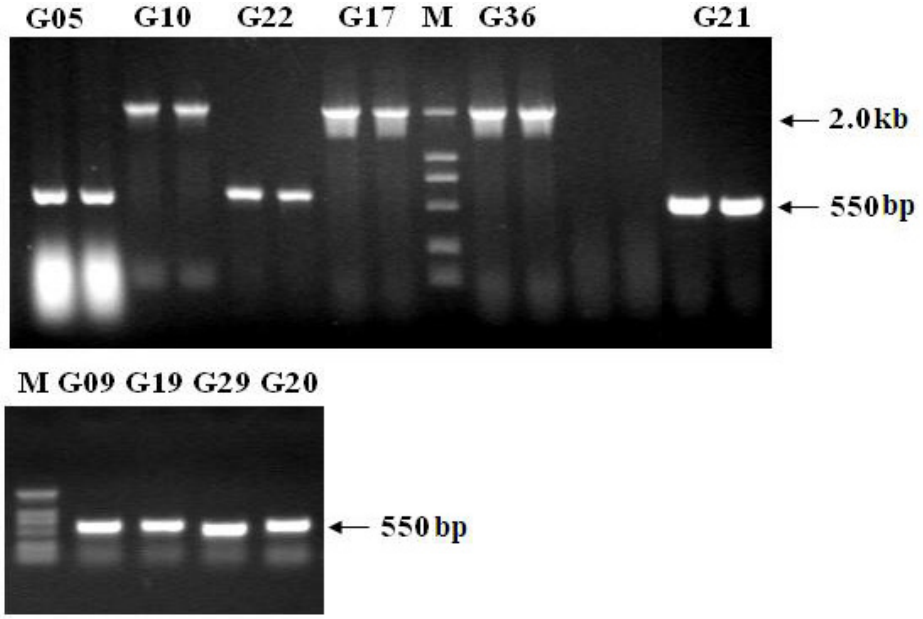

Figure 4. PCR of the mt SSU rDNA from the *Ganoderma* isolates. *Lane M* = molecular marker.

Sequence alignment

Sequence alignment of the 7 taxa was carried out. Alignments of the V4-V6 regions are included in **Figure S1**. The nucleotide sequence in the V4 region varied from 214-283 bp, with 77.4% consensus and 59.7% identity among the 7 taxa. The nucleotide sequence in the V5 region varied from 189-194 bp, with 95.1% consensus and 74.4% identity among the 7 taxa. The nucleotide sequence in the V6 region varied from 117-144 bp, with 93.9% consensus and 63.32% identity among the 7 taxa. The frequency of variable sites was different in the 3 regions, but was primarily located in the V4 region.

Phylogenetic analysis

To determine the evolutionary relationships of the 10 species between each other and with other *Ganoderma* species at the molecular level, and to identify the species, phylogenetic analysis was performed. Sequence data from the V4-V6 regions were aligned against the published sequence data, which is available from the GenBank database at the National Center for Biotechnology Information. Aligned sequences were then analyzed for evolutionary relationships using the ClustalW program (version 3.1). Sequences were also subjected to pairwise distance analysis in MEGA (Figure 5). The resulting phylogenetic tree suggested a wide range of genetic diversity among *Ganoderma* species. Interestingly, *G. lucidum* G05 and *G. eupense* G11 strains clustered into the *G. resinaceum* group. *Ganoderma* spp G22 and G29 strains clustered into the *G. lucidum* group. However, *Ganoderma* spp G19, G20, and G21 strains clustered into a single group, while the *G. lucidum* AF214475, *G. sinense*, *G. strum* G10, *G. strum* G36, and *G. sinense* G17 strains were clustered into other groups. The *G. lucidum* AF214475, *G. sinense*, *G. strum* G10, *G. strum* G36, and *G. sinense* G17 contain an intron.

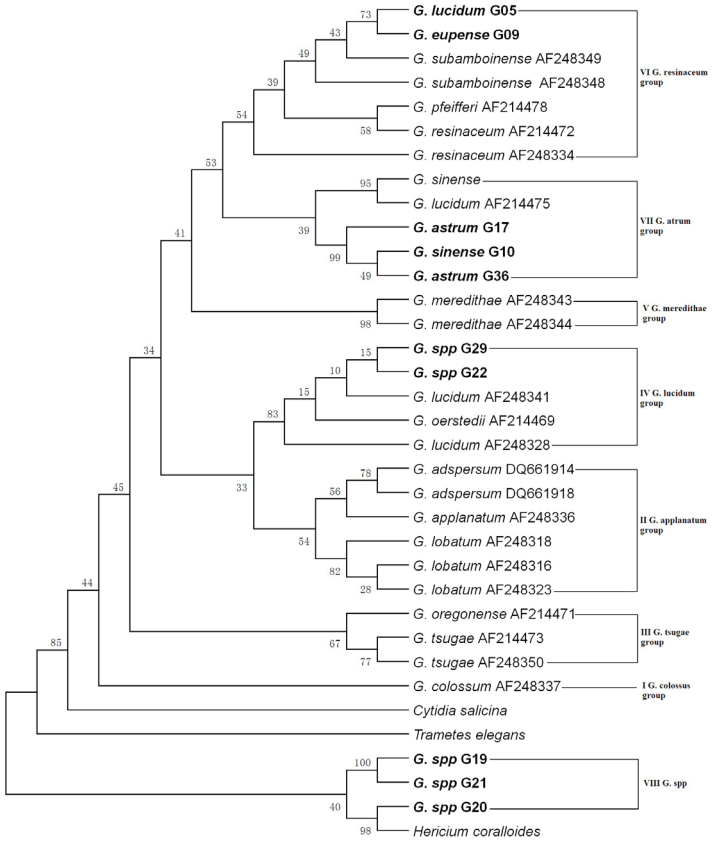

Figure 5. Phylogenetic tree indicating the relationship among isolates of *Ganoderma* based on the V4-V6 region sequence of mt SSU rDNA. The mt SSU rDNA sequences obtained from GenBank are shown with accession numbers.

DISCUSSION

Ganoderma lucidum (Ling-zhi), a widely cultivated fungus in China, has a long history of use in traditional Chinese medicine. The first record of *Ganoderma* in China was by Teng in 1934, who described 4 species and 1 variety. One of the species recorded was referred to as *G. lucidum* (Wang et al., 2012). A total of 98 species distributed throughout China were assembled by Zhao and Zhang in 'Flora Fungorum Sinicorum 18: Ganodermataceae', including 58 new species based on collections from China (Zhao and Zhang, 2000). Ling Zhi was divided into 6 types based on their different colors, such as Qing Zhi (cyan), Chi Zhi (red, *G. lucidum*), Huang Zhi (yellow), Bai Zhi (white), Hei Zhi (black), and Zi Zhi (purple, *G. sinensis*) in the ancient Chinese medical encyclopedias (Lin, 2011). Ganodermataceae contains 4 genera, including *Ganoderma*, *Amaurodama*, *Haddowia*, and *Humphreya*. *Ganoderma* consists of the subgenus *Ganoderma*, which includes Sect. *Ganoderma* and Sect. *Phaenema*, subgenus *Elfvingia*, and subgenus *Trachyderma* (Zhao and Zhang, 2000). Various collections from several provinces have been collected, including Anhui, Fujian, Guangxi, Guizhou, Hainan, Jiangsu, Sichuan, Yunnan, and Zhejiang. The name was then adapted by contemporary Chinese mycologists and used to refer to *Ganoderma* species widely cultivated in China after the 1970s (Wu and Dai, 2005). Cultivation of the species became popular in the 1980s and 1990s and the name *G. lucidum* appeared in many publications and commercial catalogs, and use of this name has increased since then. In fact, *G. lucidum* is not only incorrectly recorded in China, but has also been reported incorrectly worldwide. A variety of molecular marking methods have been used to identify cultivars of *Ganoderma*, such as random amplified polymorphic DNA (Zhao et al., 2003; Luo et al., 2005), internal transcribed sequences, restriction fragment length polymorphism analysis of PCR-amplified fragments (Luo et al., 2005; Su et al., 2007; Zhou et al., 2008), amplified fragment length polymorphism (Wu et al., 2009). PCR-restriction fragment length polymorphism shows good stability and high repeatability, which is important for phylogenetic analysis. Its resolution is high in genera and in a genus, but intraspecific polymorphism identification is not specific. Research also shows that this technique shows good resolution in different strains, but it cannot reveal differences between strains. Because a large quantity of samples can be analyzed with high sensitivity, random amplified polymorphic DNA uses the whole genome as a target and is suitable for identifying different species. However, this method shows low stability. Based on differences in fingerprints between strains, amplified fragment length polymorphism can effectively distinguish the genotypes of different strains, which may be effective for quality control when analyzing and identifying cultivated strains. Amplified fragment length polymorphism has been successfully used to identify the main cultivated species of *Ganoderma* strains, and 30 *Ganoderma* production strains from various districts of China formed a tight cluster in 8 groups (Wu et al., 2009).

In the study, 2 strains were chosen from the Agricultural Culture Collection of China as controls in order to identify 8 other strains for their taxonomic positions and medical values. The 2 controls were clustered into 2 groups, *G. resinaceum* and *G. atrum*. Compared with the traditional taxonomical system, these clustering results are in agreement with the taxonomical system. Two strains, G29 from Shandong Agriculture University and G22 from the Sichuan Academy of Agricultural Sciences, were clustered to group *G. lucidum*. The results indicated that the 2 strains had the same features and medical values. Strains G12 collected from the Agricultural Culture Collection of China, G20 collected from the Institute of Edible Fungi of Middle China, and G36 collected from the Institute of Edible Fungi, Shanghai Academy of

Agricultural Sciences were clustered to *G. atrum*, although they were collected from different sites. Sequences for the 3 strains and 2 strains were obtained from the NCBI database and contained an intron. Strain G19, which was collected from the Institute of Edible Fungi of Middle China (Wuhan), G20 collected from the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, and G21 collected from the Shaanxi University of Technology were clustered into a group. Although they belonged to *Ganoderma*, the strain G19 belong to Sect. Phaeonema, while strain G20 has a closer genetic relationship with Sect. Phaeonema using amplified fragment length polymorphism (Wu et al., 2009). Strain 21 was originally from South Korea, and although it belongs to Sect. *Ganoderma*, some variation developed after it was introduced to the area (Wu et al., 2009). The morphological characteristics and medical value of the 3 strains require further analysis.

ACKNOWLEDGMENTS

Research supported by grants from the National Natural Science Foundation of China (#30771500), the Shanghai Science and Technology Committee and the Shanghai Leading Academic Discipline Project.

Supplementary material

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