INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
15–476/2016/18–5–932–938
DOI: 10.17957/IJAB/15.0189
http://www.fspublishers.org

Full Length Article

Species Clarification of the Widely Cultivated *Ganoderma* in China Based on rDNA and *FIP* Gene Sequence Analysis

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Abstract

Among all *Ganoderma* species, only *G. lucidum* and *G. sinense*, have been approved to produce health products. Thus, it is important for *Ganoderma* production and trade to select a suitable genetic marker for *Ganoderma* species clarification. The ITS region of rDNA and *FIP* nucleotide sequences data were used for identification of twenty widely cultivated *Ganoderma* strains. The sizes of ITS1-5.8S-ITS2 region from different species of *Ganoderma* ranged from 452 to 474 bp, and those of the *FIP* nucleotide sequence were from 333 to 336 bp. Using the rDNA ITS region and *FIP* gene sequences data. and phylogenetic analysis revealed that these *Ganoderma* species belong to two groups i.e., red *Ganoderma* and black *Ganoderma*. In conclusion both the FIP and ITS1-5.8S-ITS2 genes can be used as genetic markers to distinguish two subgenera *Ganoderma*: Sect. *Ganoderma* and Sect. *Phaenema*, confirm widely cultivated *Ganoderma*, which mainly belong to subgenus *Ganoderma*. © 2016 Friends Science Publishers

Keywords: *Ganoderma*; Internal transcribed spacer (ITS), Fungal immunomodulatory protein (FIP), Molecular identification, Ribosomal DNA

Introduction

Ganoderma P. Karst. (*Basidiomycetes*, *Ganodermataceae*) is a genus of well-known medicinal mushrooms, which is called as Lingzhi in Chinese, *Ganoderma* in English, belongs to a kind of wood decaying polypore fungi of economic importance, and a widely distributed fungal genus with high diversity of species especially in the tropics (Wang and Yao, 2005; Zhou *et al*., 2007b). In the ancient China, *Ganoderma* had been regarded as a herb of longevity and used for preventing and treating diseases for more than two thousand years. Because of the unique pharmacological function and without toxic and side effects, the genus *Ganoderma* has got a good reputation in some Asian and African countries as the folk herbal medicine. Now the trade value of Lingzhi products in the world market is increasing year by year. Because of its irregular and sparse distribution in the wild state, and an increasing demand for Lingzhi as a traditional medicinal herb, attempts were made for artificial cultivation of this mushroom (Chang and Buswell, 2008). Till now, the artificial cultivation of Lingzhi has become an encouraging industry based on biotechnology in China and most other Asian countries, and is quickly spreading to other countries of the world. Actually, among more than one hundred genera of *Ganoderma*, only two species, *G. lucidum* Leyss. Ex Fr. and *G. sinense* Zhao, Xu et Zhang, have been recorded not only in the Chinese Pharmacopoeia but also in the American Herbal Pharmacopoeia and Therapeutic Compendium (Zhou *et al*., 2012). A variety of Lingzhi products with various bioactive ingredient are available on the market, such as spore powder, fruiting bodies, mycelia or (and) their extracts, which were processed into capsule, tablet or other type of products (Zhou *et al*., 2012; Ogbe *et al*., 2009). The growth and artificial cultivation conditions of different members of the family *Ganoderma taceae* was different (Mayzumi *et al*., 1997). Moreover, different *Ganoderma* species are favored in different geographical regions with different climatic conditions. For example, *Ganoderma* with the black color is popular in southern China, whereas *Ganoderma* with the red color is preferred in Japan. Therefore, the precise identification and classification of *Ganoderma* and its cultivation source used for industrial and commercial purposes is very important.

With the development of molecular biology, some new techniques have been applied to fungal classical taxonomy. DNA markers that those could be obtained by sequencing methods, are reliable for informative genetic polymorphisms. There are various mature molecular marker techniques that had been widely applied in the classification

To cite this paper: Zhou, X.W., Y. Liu, M.Y. Guo, K.Q. Su and Y.M. Zhang, 2016. Species clarification of the widely cultivated *Ganoderma* in China based on rDNA and *FIP* gene sequence analysis. *Int. J. Agric. Biol.,* 18: 932–938

of *Ganoderma* strains, such as the encoding regions of the 18S, 5.8S, 28S and ITS regions of nucleotide sequences of the rDNA (Moncalvo *et al*., 1995; Gottlieb *et al*., 2000; Smith and Sivasithamparam, 2003; Lee *et al*., 2006; Sun *et al*., 2006; Zheng *et al*., 2009) and mt SSU rDNA (mitochondrial small subunit rDNA) (Hong *et al*., 2002; Hong and Jung, 2004), etc.

With the development of cloning and sequencing techniques, the abundant information on functional genome sequences in fungi is available in some open databases, which makes it possible to develop new marker systems. These protein-coding genes have genus-specific, species-specific and strain-specific characters. For example, MnSOD-based phylogenetic analysis of 28 *Ganoderma* isolates of the *G. lucidum* complex exhibited five groups, like in ITS- and LSU rRNA-based analysis (Fréalle *et al*., 2005); fungal immunomodulatory protein (FIP)-based identification can provide a valid evidence in molecular level distinction to distinguish the genuine origin of *G. lucidum*, *G. sinense* and *G. tsugae* (Zhou *et al*., 2008) and partial β-tubulin gene could also be used for distinguishing *G. lucidum* strain isolated in Korea from other *G. lucidum* (Park *et al*., 2012).

The cultivated *Ganoderma* species complex in China includes *G. lucidum*, *G. tsugae*, *G. japonicum*, *G. sinenes*, *G. tenus*, *G. atrum*, *G.* spp. and several other groups that are only found in the tropical areas. Among the genus of *Ganoderma*, more than ten species are used in production, but only two species can be used for production of drug or health care products. These *Ganoderma* species commonly come from China, Japan and Korea. In an overview of previous literature, there is much confusion as to which is the true *Ganoderma* species (Zhou *et al*., 2012). The Japanese thought that the true *Ganoderma* should be with red color, *Ganoderma* species with a different colour was due to variations in its growing environmental conditions which included temperature, humidity, light condition, etc. The Chinese believed that the true Lingzhi should be with black color because there are a lot of reports about black Lingzhi that had unusual medicinal benefits (Mayzumi *et al*., 1997). However, setting up an accurate identification method of *Ganoderma* or a phylogenetically-based classification system, together with developing novel genetic markers for individual strains, would have potential applications in further pharmacological studies. In the present study, we collected twenty cultivated *Ganerdama* species, cloned the nucleotide sequences of ITS1-5.8S-ITS2 and FIPs. Using these nucleotide sequences, we also generated phylogenetic characters and analyzed evolutionary relationship between each *Ganoderma* strains. The ITS dataset would offer undreamed-of results on phylogenetic information at high taxonomic levels based on this new design and analysis. In addition, we can also specifically identified the *Ganoderma* species as differing from China, Japan and Korea using the ITS rDNA region. The results of this study could 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 the genus of *Ganoderma*.

Materials and Methods

Ganaderma Materials

The twenty species of *Ganoderma* used in this study were obtained from different provinces of China. These fungal specimens included some commercial cultivars and wild species, which were presented as follows: *G. lucidum* (01-07) from Microbial Culture Collection Center, Guangdong Institute of Microbiology (Guangdong, China); *G. japonicum* (08), *G. sinense* (09), *G. tenus* (10) and *G. lucidum* (11) from Agricultural Culture Collection of China (ACCC); *G.* spp.(12, 13) and *G. atrum* (14) from Edible Fungi Cultivation Institute of Huazhong District (Wuhan, China); *G.* spp. (15) from Sichuan Academy of Agricultural Sciences (Chengdu, China); *G.* spp. (16) from Heilongjiang Academy of Agricultural Sciences (Harbin, China); *G.* spp. (17) from Shandong Agriculture University (Taian of Shandong, China); *G.* spp. (18, 19) and *G. atrum* (20) from Edible Fungi Institute, Shanghai Academy of Agricultural Sciences (Table 1). *Ganoderma* mycelia were prepared by liquid culture methods in our laboratory.

Preparation of *Ganoderma* Mycelia

The *Ganoderma* strain was stored at 4°C on PDA (potato dextrose agar) slants, containing potato 200 g, dextrose 20 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, KH_2PO_4 2.5 g, vitamin B_1 10 mg, agar 20 g per liter of water. Stock cultures were plated onto the petri dishes containing PDA and allowed to incubate for 5 d at 28°C. Two agar blocks (φ9mm) were obtained and transferred into a 250 mL Erlenmeyer flask containing 50 mL of fresh liquid state medium, consisting of sucrose 35 g, peptone 5 g, yeast extract 2.5 g, $MgSO_4$ 0.5 g, KH_2PO_4 1 g, vitamin B_1 50 mg per liter of water, initial pH 6.8. The *Ganoderma* mycelia were incubated on a rotary shaker (150 rpm, 5~10 d, and 28°C) and harvested by centrifugation (5000 × *g*, 15 min). After being washed thrice with PBS buffer, the mycelia were gathered by centrifuged, respectively, and then treated with dry refrigeration for extraction of genomic DNA.

DNA Extraction

The DNA isolations were performed using the method as described by Zhou *et al*., (2007a). Mycelia (1 g) were ground in liquid nitrogen, then transferred to a 50 mL tube containing 5.0 mL of extraction buffer and mixed gently. The samples were then placed in a water bath at 65°C for 30 min. The DNA-particulate fraction was pelleted by centrifugation at 10,000 × g for 10 min and the aqueous

phase was carefully transferred into a fresh tube containing 2/3 volume 2.5 M kalium aceticum (pH 4.8) solution. The tube was shaken gently, and then maintained at 0°C for 30 min until the protein were precipitated. Proteins were pelleted by centrifugation at 4°C and 10,000 × g for 10 min and the aqueous phase was transferred into a fresh tube containing 2/3 volume cold isopropanol, then maintained at -20°C for at least 16 h. After centrifugation, the pellet was collected, then washed with 70% ethanol and resuspended in 500 μL TE buffer. The DNA was stored at -20°C.

PCR Amplification

The reaction mix for PCR amplification of TS1-5.8S-ITS2 rDNA region and FIPs gene consisted of 50 μL volume, 0.25 mM each of the primer pair, 0.25 mM deoxy-ribonucleoside triphosphate, 1.5 mM magnesium chloride, 5μL of a 1/100 dilution of the DNA extraction, 1 U of rTaq polymerase (TaKaRa, China) and 10× PCR buffer mix (TaKaRa, China). Two primers (ITSF: 5'-GGGTTGTAGCTGGCCTTCCGAGGC ACGT-3' and ITSR: 5-TTATCACACCGAGAGCCGATCCGCAAGG-3') were used for amplification of the ITS1-5.8S-ITS2 region nucleotide sequence, and two primers (FIPA: 5'-ATGTCCGACACTGCCTTGATCTTCAGG-3' and FIPB: 5'-CTAGTTCCACTGGGCGATGATGAAGTC-3') were used for amplification of the *FIPs* nucleotide sequence. The PCR reactions were performed using a PTC-100™ programmable thermal controller (MJ Research, Inc, UK) for 30 cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 40 sec) followed by extension for 10 min at 72°C. A 5 μL aliquot of each sample was mixed with 0.5 μL of ethidium bromide, then electrophoresed on a 1.5% agarose gel, and visualized with a UV transilluminator.

Cloning and Sequencing

The PCR products were purified using a multifunction DNA purification KIT (BioTeke Inc.), ligated into the pMD18-T vectors (Takara, Dalian, China), transformed into DH5α competent cells (*E. coli*) and then sequenced by Sangon Biotech (Shanghai, China) Co., Ltd.

Sequence Alignment and Analysis

Sequence data was used to make a comparison with some isolates used in this study, are listed in Table 1. DNA sequences were edited and aligned using the ClustalW multiple sequence alignment programs at the Biology Workbench Version 3.2 (Thompson *et al*., 1994). Final multiple alignments were visually checked and adjusted manually when necessary. Phylogenetic analysis was conducted by the ITS1-5.8S-ITS2 region and FIPs sequences using the Molecular Evolutionary Genetics Analysis (MEGA) software version 2.1 (Tamura *et al*., 2011).

Results

DNA Sequence Amplifications

The primer pair ITSF/ITSR was used for amplification of the ITS1-5.8S-ITS2 region sequences. The isolates yielded product sizes of variable lengths, from 452 to 474 bp (Fig. 1A). The primer pair FIPA/FIPB was used for amplification of FIPs gene sequences, the isolates yielded fragments of about 330~3336 bp (Fig. 1B).

Sequence Analysis

A rDNA repeat unit contained the ITS (internal transcribed spacer) regions and intergenic spacer (IGS) regions of *G. lucidum* (Fig. 2). The PCR amplified ITS products contained ITS I 188 bp, 5.8S rDNA 158 bp, ITS II 125 bp. The sizes of PCR product from the ITS regions and FIP genes were close to those investigated by (Zhou *et al*., 2009) and (Moncalvo *et al*., 1995), respectively. Sequence alignment of the twenty taxa was performed using the ClustalW multiple sequence alignment programs at the Biology Workbench Version 3.2 (Thompson *et al*., 1994). There were two most variable areas in ITS I and ITS II region (Fig. 3A), corresponding to the 70~144 and 350~400 regions of the rDNA. Nucleotide sequence in the ITS I region varied in length from 176 bp to 188 bp, with 98.9% consensus positions and 67.6% identity positions. Nucleotide sequence in the ITS II region varied in length from 121 bp to 125 bp, with 96.0% consensus positions and 81% identity positions. The frequency of variable sites was different in the ITS1 and ITS2 regions, but was mostly located in the ITS I region (Fig. 3A). The 5.8S gene that located between the ITS I and II regions was very well conserved (158 bp in length). The 5.8S rDNA sequences of the basidiomycetes fungi were identical, which was a result agreeing with our findings (Sun *et al*., 2006). Nucleotide sequence in the FIPs gene varied in length from 333 bp to 336 bp, with 100% consensus positions and 77.1% identity positions (Fig. 3B). The sequence information for the ITS region and *FIP* gene was shown in Table 2. In the ITS region total G+C and A+T content varied from 47.3~48.7% and 51.3~52.7%, respectively; or in the ITS region varied from 61.0~58.9% and 39.0~41.1%, respectively. Final sequence alignments were visually checked and manually optimized. All the fungal species, genus, family and class had specific G+C content, close genetic relationship between materials with similar G+C content (Jia *et al*., 2003). The results showed that G+C content of FIP gene was closest among G05, G09 and G14 strains, between 60.1%~60.4%, the remaining seventeen strains were closest, ranging between 58.9%~59.5%. There were small changes in the G+C content of ITS region gene in all strains, in addition to G09 strains.

Table 1: *Ganoderma* species used in the present study

No.	Species	Collection sites	Collection sites	Origin
1	*G. lucidum*	GIM 5.8	Microbial Culture Collection Center of Guangdong Institute of Microbiology	China
2	*G. lucidum*	GIM 5.9		China
3	*G. lucidum*	GIM 5.11		China
4	*G. lucidum*	GIM 5.257		China
5	*G. lucidum*	GIM 5.259		China
6	*G. lucidum*	GIM 5.252		Japan
7	*G.* spp.	GIM 5.253		Korea
8	*G. japonicum*	50045	Agricultural Culture Collection of China, ACCC	China
9	*G. sinensis*	51229		China
10	*G. tenus*	50604		China
11	*G. lucidum*	50088		China
12	*G.* spp.	HZ1	Edible Fungi Cultivation Institute of Huazhong District	Japan
13	*G.* spp.	HZ2		China
14	*G. atrum*	HZ3		China
15	*G.* spp.	SH1	Sichuan Academy of Agricultural Sciences	Japan
16	*G.* spp.	HLJ1	Heilongjiang Academy of Agricultural Sciences	Korea
17	*G.* spp.	SD1	Shandong Agricultural University	China
18	*G.*spp.	SH1	Shanghai Academy of Agricultural Sciences	Korea
19	*G.* spp.	SH2		China
20	*G. atrum*	SH3		China

Table 2: Sequence information of ITS and the FIP gene sequence of *Ganoderma* species

No.	Species	Collection sites	Internal transcribed spacer rDNA			FIP gene		
			Length (bp)	A+T content (%)	G+C content (%)	Length (bp)	A+T content (%)	G+C content (%)
1	*G. lucidum*	GIM 5.8	468	51.5	48.5	336	41.1	58.9
2	*G. lucidum*	GIM 5.9	468	51.3	48.7	336	40.5	59.5
3	*G. lucidum*	GIM 5.11	468	51.5	48.5	336	40.9	59.1
4	*G. lucidum*	GIM 5.257	468	51.5	48.5	336	41.1	58.9
5	*G. lucidum*	GIM 5.259	472	51.5	48.5	336	39.0	61.0
6	*G. lucidum*	GIM 5.252	468	51.3	48.7	333	40.8	59.2
7	*G.* spp.	GIM 5.253	468	51.5	48.5	336	40.8	59.2
8	*G. japonicum*	50045	468	51.5	48.5	336	41.1	58.9
9	*G. sinensis*	51229	473	52.9	47.1	336	39.6	60.4
10	*G. tenus*	50604	468	51.9	48.1	336	41.1	58.9
11	*G. lucidum*	50088	469	51.8	48.2	336	41.1	58.9
12	*G.* spp.	HZ1	468	51.9	48.1	336	41.1	58.9
13	*G.* spp.	HZ2	468	51.5	48.5	336	41.1	58.9
14	*G. atrum*	HZ3	472	51.7	48.3	336	39.9	60.1
15	*G.* spp.	SH1	468	51.5	48.5	336	41.1	58.9
16	*G.* spp.	HLJ1	468	51.7	48.3	336	41.1	58.9
17	*G.* spp.	SD1	468	51.5	48.5	336	41.1	58.9
18	*G.*spp.	SH1	468	51.5	48.5	336	41.1	58.9
19	*G.* spp.	SH2	452	51.3	48.0	336	41.1	58.9
20	*G. atrum*	SH3	472	51.9	48.1	336	41.1	58.9

Phylogenetic Analyses

Phylogenetic relationship analysis was performed using the MEGA software version 2.1 (Tamura *et al*., 2011). The phylogenetic trees constructed from the specific nucleotide sequences of the ITS1-5.8S-ITS2 region and FIP gene showed in Fig. 4A and Fig. 4B, respectively. The results of phylogenetic tree analysis showed that a greater level of genetic diversity of *Ganoderma* species originated from different geographical regions. All the strains were clustered into two groups using FIP gene sequence (Fig. 4A), strains 05, 10, 17 and 36 had the same sequence identity, were clustered to sect. Phaeonema; and other sixteen strains were clustered to sect. *Ganoderma*. The results were identical to classical taxonomy and molecular taxonomy (Zhao and Zhang, 2000); all the strains were clustered into six groups using ITS region sequence (Fig. 4B). Strains 05, 17, 32, 36 were clustered to a subgroup; strain 9 was clustered to a subgroup, other fifteen strains were clustered to four subgroups. The clustering results of strains 05, 17, 36 coincided with the identification using FIP sequence. The clustering result of strain 32 was different using ITS and FIP regions sequences. The rest fifteen strains were clustered into one subgroups using FIP gene sequence, but these strains were clustered into four subgroups using ITS region sequence, these results showed that ITS region sequence was suitable for using as a marker for species differentiation. Six strains of *Ganoderma* collected from China were clustered into groups I, three strains from Korea were clustered into groups II, three strains from Japan were clustered into groups I, III and IV, respectively.

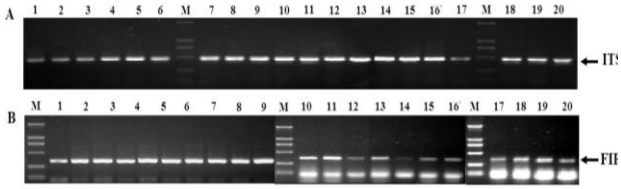

Fig. 1: Results of PCR amplification of a ITS gene (A) and FIP gene (B) for twenty selected *Ganoderma* strains. MW: molecular weight marker (DL2000)

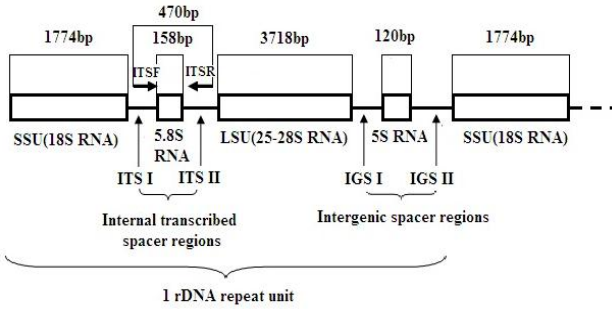

Fig. 2: A ribosomal DNA repeat unit showing the internal transcribed spacer regions and intergenic spacer regions. The diagram shows the location of the ribosomal subunits within the repeat unit. The diagram displays the orientation and location of oligonucleotides primers used during the PCR amplification in the investigation

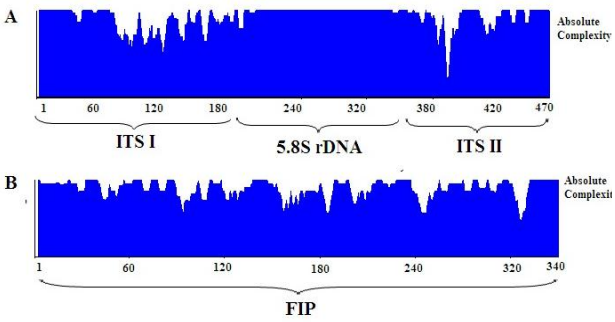

Fig. 3: Aligmnet of variable domains (ITS, FIP) are shown using Vecter NTI

The resulting phylogenetic tree showed a greater level of genetic diversity between *Ganoderma* species originating from different geographical regions.

Discussion

G. lucidum was first introduced to China by Teng (1934) for sampling from several provinces of China i.e., Fujian, Jiangsu, Guangxi, Anhui, Sichuan, Guizhou, Hainan, Yunnan, Zhejiang and etc. This name was then accepted by Chinese mycologists (Chow and Chen, 1935) and was used for the Lingzhi cultivation in China after the 1970s, then became more and more popular from 1980s to 1990s (Tai, 1979; Zhao, 1989; Hong and Jung, 2004; Wu and Dai, 2005). Currently, the amount of demand about *G. lucidum* is very large in the world. In 1997, the *G. lucidum* production was 4300 tons in the world, of which China accounted for 3000 tons. In 1999, it had been reported that the annual production value of *G. lucidum* products in the world was 1628.4 million U.S. dollars, in China for 350 million U.S. dollars, and in 2000, the annual production values of Lingzhi products achieved more than 2.16 billion U.S. dollars (Xiao *et al*., 2006). However, cultivated Lingzhi varieties were not only incorrectly recorded throughout China, but were similarly reported incorrectly from all around the world. The name of *G. lucidum* has been applied to collections from North America, East Africa, South America and Southeast Asia, East Asia, Oceania as well as Europe (Wang *et al*., 2012). The collections named as *G. lucidum* from different regions of the world have appeared in several separated taxa in phylogenetic biogeographic analyses of the genus *Ganoderma* (Gilbertson and Ryvarden, 1986; Moncalvo *et al*., 1995; Gottlieb *et al*., 2000; Hong and Jung, 2004; Lee *et al*., 2006). It is obvious that those collections were identified incorrectly, especially if they were not sampled from Europe, and were in need of further taxonomic studies.

The traditional taxonomy of *Ganoderma* is based on its morphological traits, such as front cap color with a lacquer-like sheen, color of context, crust structure, basidiospore ornamentation and etc. *Ganodermataceae* contains four genera: *Ganoderma*, *Amaurodama*, *Haddowia* and *Humphreya*. *Ganoderma* consists of subgenus *Ganoderma* that includes sect. *Ganoderma* and sect. *Phaenema*, subgenus *Eflvingia* and subgenus *Trachyderma* (Zhao and Zhang, 2000). Additionally, the observed differences in morphological character could be resulting from simple mutations or cultivation effects and thus were not always reliable identification characters. The limitations of morphological identification techniques indicate that we should develop reliable alternative method for the identification of *Ganoderma*. Now, a variety of sequence-based DNA fingerprinting techniques have been used to the identification of cultivation of *Ganoderma*, such as internal transcribed spacer (ITS) 25S ribosomal DNA sequencing PCR amplification (Moncalvo *et al*., 1995; Gottlieb *et al*., 2000; Hong and Jung, 2004; Sun *et al*., 2006), PCR-RFLP (Zhou *et al*., 2008; Park *et al*., 2012), sequence characterized amplified region (Xu *et al*., 2008) and sequence-related amplified polymorphism molecular marker system (Sun *et al*., 2006). The use of these techniques is dependent on the ribosomal RNA sequences. Taxonomy of China widely cultivated *Ganoderma* has been studied by researchers for many decades (Zhao *et al*., 2003; Luo *et al*., 2005a, b; Tang *et al*., 2005a, b; Su *et al*., 2007; Wu *et al*., 2009; Zhou *et al*., 2008; Gao *et al*., 2010; Huang *et al*., 2010; Cao *et al*., 2012; Wang *et al*., 2012),

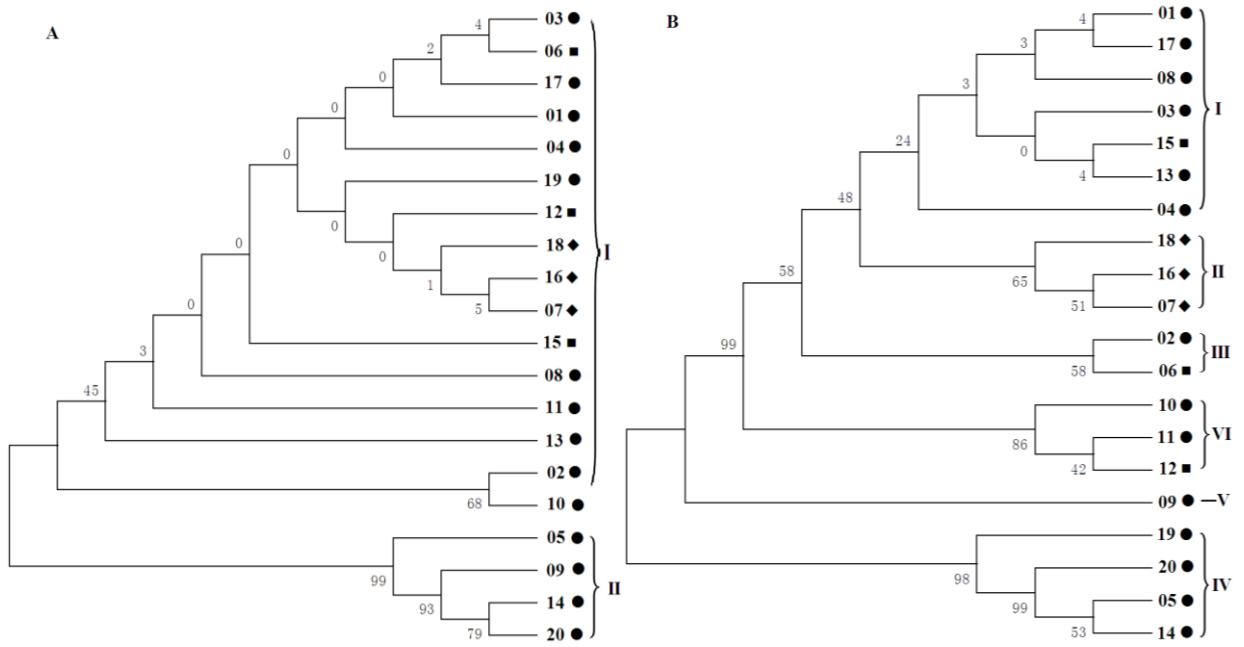

Fig. 4: Phylogenetic trees constructed from the FIP (A) and ITS regions sequence (B) of the *Ganoderma* species. ● *G. lucidum* (China); ■ *G. lucidum* (Japan);◆ *G. lucidum* (Korea)

but numerous taxonomic problems remain and the molecular taxonomy of China widely cultivated *Ganoderma* has not previously been addressed. Intuitive species boundaries, phylogenetic relationships, distribution and host range of taxa of the *G. lucidum* complex are really not clear even among the few taxa nowadays recognized in China. The distributions of *Ganoderma* in China mainly contain subgenus *Ganoderma* and subgenus *Eflvingia*, only three species belong to the subgenus *Trachyderma* (Jia *et al*., 2003). The widely cultivated *Ganoderma* in China mainly belong to subgenus *Ganoderma*. Many researchers have been shown the genetic diversity between the subgenus *Elfvingi* and subgenus *Ganoderma* is rich, but a smaller degree of genetic diversity among Sect. *Ganoderma*.

Conclusion

The *FIP* and ITS1-5.8S-ITS2 nucleotide sequences can be used as molecular markers to distinguish two subgenera *Ganoderma*: Sect. *Ganoderma* and Sect. *Phaenema*, confirm widely cultivated *Ganoderma* in China which mainly belong to subgenus *Ganoderma*.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (30771500), Shanghai Science and Technology Committee.

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(Received 04 May 2015; Accepted 23 July 2015)