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Full Length Research Paper

AFLP analysis of genetic diversity in main cultivated strains of *Ganoderma* spp.

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Ganoderma **mushroom is one of the most prescribed traditional medicines, which has been used for medicinal purposes for centuries particularly in China, Japan, Korea and other Asian countries. In this article, the different strains of** ***Ganoderma*** **spp. used in production and their genetic relations of the closely related strains were identified and investigated at molecular level. The 30** ***Ganoderma*** **strains were collected and analyzed using amplified fragment length polymorphism (AFLP) techniques. By using 8 primer combinations, the results showed that there were 52 polymorphic AFLP markers, and all accessions could be uniquely identified. Among the** ***Ganoderma*** **accessions, similarity coefficients ranged from 0.3282 to 0.746 in AFLP. The** ***Ganoderma*** **strains formed a tight cluster in eight groups in AFLP. Herein, among the collected** ***Ganoderma*** **spp.,** ***Ganoderma*** **(from Korea) had some variations after introduction from original sites. These variances are related to different ecological habitats, and caused subtle discrimination in morphological traits and medical value. AFLP as a tool to identify the** ***Ganoderma*** **species or cultivars are very sensitive and practical.**

Key words: *Ganoderma* spp., AFLP technique, identification, molecular marking.

INTRODUCTION

Ganoderma, for its perceived health benefits, has gained wide popularity as a dietary supplement in China, Japan, Korea and other regions of the world (Zhou et al., 2007b). For a long time past, classification of *Ganoderma* mushroom is based on morphological characteristics, which is often highly subjective, with unambiguous identification to the species level often being extremely difficult. Additionally, the morphological differences observed may be the product of simple mutations or media/cultivation effects and thus are not always reliable characters. It limited the accuracy of identification, and the genetic variety of different strains cannot be objectively described. In production, some strains identification and circumscription were often unclear and taxonomic segregation of the genus remained controversial, and even a number of *Ganderma* strains have been misnamed. The limitations of traditional identification techniques indicate that alternative methods need to be developed for the identification of these fungi. With the development of molecular biology, some new techniques have been applied to fungal classical taxonomy. DNA fingerprinting techniques, however, would be allowed to identify the *Ganoderma* species and cultivars, indicating that it is a useful tool for the valid protection of newly bred cultivars. A variety of laboratory-based techniques have been used to study genetic diversity in *Ganoderma*, such as isozyme analysis (Lan et al., 1998), random amplified polymorphism DNA (RAPD) (Tang et al., 2005; Hseu et al., 1996), amplified fragment length polymorphism (AFLP) fingerprinting (Qi et al., 2003), internal transcribed spacers (ITS) 25S ribosomal DNA sequencing technique (Moncalvo et al., 1995a and 1995b; Gottlieb et al., 2000; Hong and Jung, 2004), PCR-RFLP (Park et al., 1996; Zhou et al., 2008), sequence characterized amplified region (SCAR)(Xu et al., 2008) and sequence related amplified polymorphism (SRAP) (Sun et al., 2006).

Isozymes are generally limited by the relatively low

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levels of detectable polymorphism and might fail to identify cultivars which differ in only a few genes (Jarret and Litz, 1986). PCR-based RAPD assay overcomes many of the technical limitations of RFLPs but has been proved to be so sensitive to experimental conditions (Paul et al., 1997). ISSR markers are more reliable than RAPD but, they have been proved to be less polymorphic and efficient than RAPD (Zhou et al., 2004). The AFLP technique is based on the PCR amplification of a fraction of restriction fragments generated by the digestion of total DNA (Vos et al., 1995), and has merits of RFLP and RAPD (Zabeau and Vos, 1993; Vos et al., 1995). AFLP fingerprinting was used as a tool in biodiversity studies, analysis of germplasm collection, genotyping of individuals, genetic distance analysis and genetic mapping in many kinds of organisms, including edible medicinal fungi, such as *Pleurotus ostreatus* (Meng et al., 2003), *Tricholoma matsutake* (Chen et al., 2003), *Lentinula edodes* (Zhuo et al., 2006), *Agaricus bisporus* (Gu et al., 2003), *G. lucidum* (Zheng et al., 2007). The AFLP technique has wide application prospect in molecular biology, genetics and breeding for edible medicinal mushroom.

In this work, AFLP technique was used for the genetic study of thirty *Ganoderma* strains. The aim of this paper was to lay a foundation for the establishment of genetic database, which could provide molecular evidence for selection and breeding of eminent cultivars and genetic relationship in *Ganoderma*.

MATERIALS AND METHODS

Ganoderma strains and preparation of the *Ganoderma mycelium*

In this study, 30 *Ganoderma* strains were collected from various units which include universities and special institution and contributing everywhere in China (Table 1). The strains and mycelium were inoculated on PDA medium (15 g/L pollard extract, 2 g/L peptone, 2 g/L yeast, 20 g/L glucose, 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ and 2.1% agar) and incubated at 25°C for 1 week. Next, the strains were inoculated in liquid medium (glucose 40 g, peptone 10 g, NaCl 2.5 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g, bringing the total volume to 1000 mL with distilled water) and incubated at 26°C for 10 d. And last, the mycelium was dried with filter paper and then stored at -20°C.

DNA extraction

Ganoderma DNA was extracted using the modified CTAB method (Zhou et al., 2007a). 0.1 g mycelium samples were ground with liquid nitrogen followed by the addition of 800 µL extraction buffer (2% w/v CTAB, 100 mmol/L Tris-HCl, 20 mmol/L EDTA, 1.4 mol/L NaCl, 7%v/v β-mercaptoethanol and pH 8.0). After being incubated for 30 min at 60°C, the homogenate was extracted with an equal volume of chloroform/ isoamyl alcohol (24:1) and centrifuged at 12,000 rpm for 15 min. The supernatant was recovered and incubated with 1.5 volume of precipitation buffer (1% w/v CTAB, 0.05 mol/L Tris-HCl, 0.01 mol/L EDTA, pH 8.0) for 30 min at room temperature, and centrifuged at 12,000 rpm to obtain DNA sedimentation. Next, the sediment was dissolved in high-salt TE buffer (10 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L EDTA (pH 8.0), 1 mol/L NaCl)) and 1µL of RNase A (2.5 U/ml) was added to the solution at 37°C for 30 min to digest RNA. Then the DNA was precipitated by 2.5 volume absolute ethanol and 1/10 volume 3 mol/L NaAC for 1 h at -20°C. The ethanol precipitation was then washed with 70% ethanol, dried and resuspended in 100 µL of TE buffer. DNA concentrations were estimated and standardized against the known concentrations of DNA on 1.5% (w/v) agarose gels. Aliquots from the DNA preparations were used for AFLP analyses.

AFLP reaction

The AFLP analysis was carried out according to previously reference (Vos et al., 1995) with minor modifications. Restriction digests of genomic DNA with *EcoR*I and *Mse*I were carried out at 37°C for 4 h. Following heat inactivation of the restriction endonucleases, the genomic DNA fragments were ligated to *EcoR*I and *Mse*I adapters overnight at 15°C to generate template DNA for amplification. PCR was performed in two consecutive reactions. (1) The template DNA generated was first pre-amplified with AFLP primers, each having zero selective nucleotide. Pre-amplification cycle profile was as follows: incubated at 72°C for 2 min, then cycled 30 times (denatured at 94°C for 30 s, annealed at 56°C for 30 s, extended at 72°C for 1 min) and at last extended at 72°C for 3 min. (2) The PCR products of the pre-amplification were then used as template for selective amplification using two AFLP primers, each containing three selective nucleotides. The primers and adapter sequences are shown in Table 2. Selective amplification was performed as follows: one cycle of 94°C for 2 min, 65°C for 30 s and 72°C for 2 min, followed by 13 cycles with annealing temperature decreasing by 0.7°C each cycle starting with 94°C for 30 s, 65°C for 30 s and 72°C for 1 min and ended with 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. AFLP fragments were amplified with an *EcoR*I primer and an *Mse*I primer. *EcoR*I and *Mse*I primers were combined as follows:

EcoR I-AAC/ *Mse* I -CTC *EcoR* I -AAC/ *Mse* I -CTG
EcoR I -AAG/ *Mse* I -CAC *EcoR* I -AAG/ *Mse* I -CAG
EcoR I -AAG/ *Mse* I -CTT *EcoR* I -ACA/ *Mse* I -CAG
EcoR I -ACA/ *Mse* I -CTC *EcoR* I -ACT/ *Mse* I -CAA

ABI 377 automated sequencer (Applied Biosystems, Inc.) was used to analyze AFLP polymorphism; the primers of *Mse*I were labeled by FAM for detection. Gel electrophoresis was analyzed with the software GENESCAN3.1 (Applied Bio-systems, Foster city, CA, USA), and the size of sequences was analyzed with the software Binthere developed by N. Garnhart (University of New Hampshire) and available at http://tilapia.unh.edu/AFLP/BinThere.html; the date converted into XLS (Garnhart, 2001). AFLP experiments were repeated three times from DNA extraction to data analysis to test its reproducibility.

Date analysis in AFLP

For the diversity analysis, the data of XLS were converted as present (1) or absent (0) and used as a raw data matrix. A square symmetric matrix of genetic distance was obtained using Jaccard's coefficient. The dendrogram was then generated by UPGMA (unweighted pairgroup method with arithmetic average)(Sneath and Sokal, 1973) with the software DPS V7.05 (Hang et al., 2004).

RESULT

AFLP profiles of Ganoderma strains

To test the reproducibility of this experiment, three repeti-

Table 1. Strains used for AFLP analysis.

Number	Species	Collection sites
c1	*G. lucidum*	Microbiological culture and collection center of Guangdong Institute of Microbiology
c2	*G. lucidum*	
c3	*G.. lucidum*	
c4	*G.. lucidum*	
c5	*G. lucidum*	
c6	*G. lucidum*	
c7	*G. sp.*	
c8	*G. japonicum*	Agricultural Culture Collection of China, ACCC
c9	*G. eupense*	
c10	*G. sinensis*	
c11	*G. tenus*	
c12	*G. lucidum*	
c13	*G. sp.*	Central China Institute of Edible Fungi Culture
c14	*G. sp.*	
c15	*G. sp.*	
c16	*G. sp.*	
c17	*G. atrum*	
c18	*G. sp.*	
c19	*G. applanatum*	
c20	*G. sp.*	Shanghai Academy of Agricultural Sciences
c21	*G. sp.*	Shaanxi university of Technology
c22	*G. lucidum*	Sichuan Academy of Agricultural Sciences
c23	*G. ahmadii*	
c24	*G. sp.*	Heilongjiang Academy of Agricultural Sciences
c25	*G. sp.*	Shandong Agricultural University
c26	*G. sp.*	
c27	*G. sp.*	
c28	*G. sp.*	
c29	*G. lucidum*	
c30	*G. tsugae*	ACCC

Table 2. The primers and adapter sequences used in the AFLP reaction.

Primer	Primer sequence (5'→ 3')
*EcoR*I adapter 1	5'-CTCGTAGACTGCGTACC-3'
*EcoR*I adapter 2	5'-AATTGGTACGCAGTCTAC-3'
*Mse*I adapter 1	5'-GACGATGAGTCCTGAG-3'
*Mse*I adapter 2	5'-TACTCAGGACTCAT-3'
*EcoR*I pre-amplified primer	5'-GACTGCGTACCAATTCA- 3'
*Mse*I pre-amplified primer	5'-GATGAGTCCTGAGTAAC-3'

tions were used from DNA extraction to data analysis. All results could be reproducible. 8 primer combinations on 30 strains yielded total 52 polymorphic bands. Among the *Ganoderma* accessions, similarity coefficients ranged from 0.3282 to 0.746 in AFLP. The AFLP profiles obtained were able to distinguish different cultivars or species by their AFLP patterns, and results showed that they had the best fingerprinting using the primer pairs of *EcoR*I-AAC/

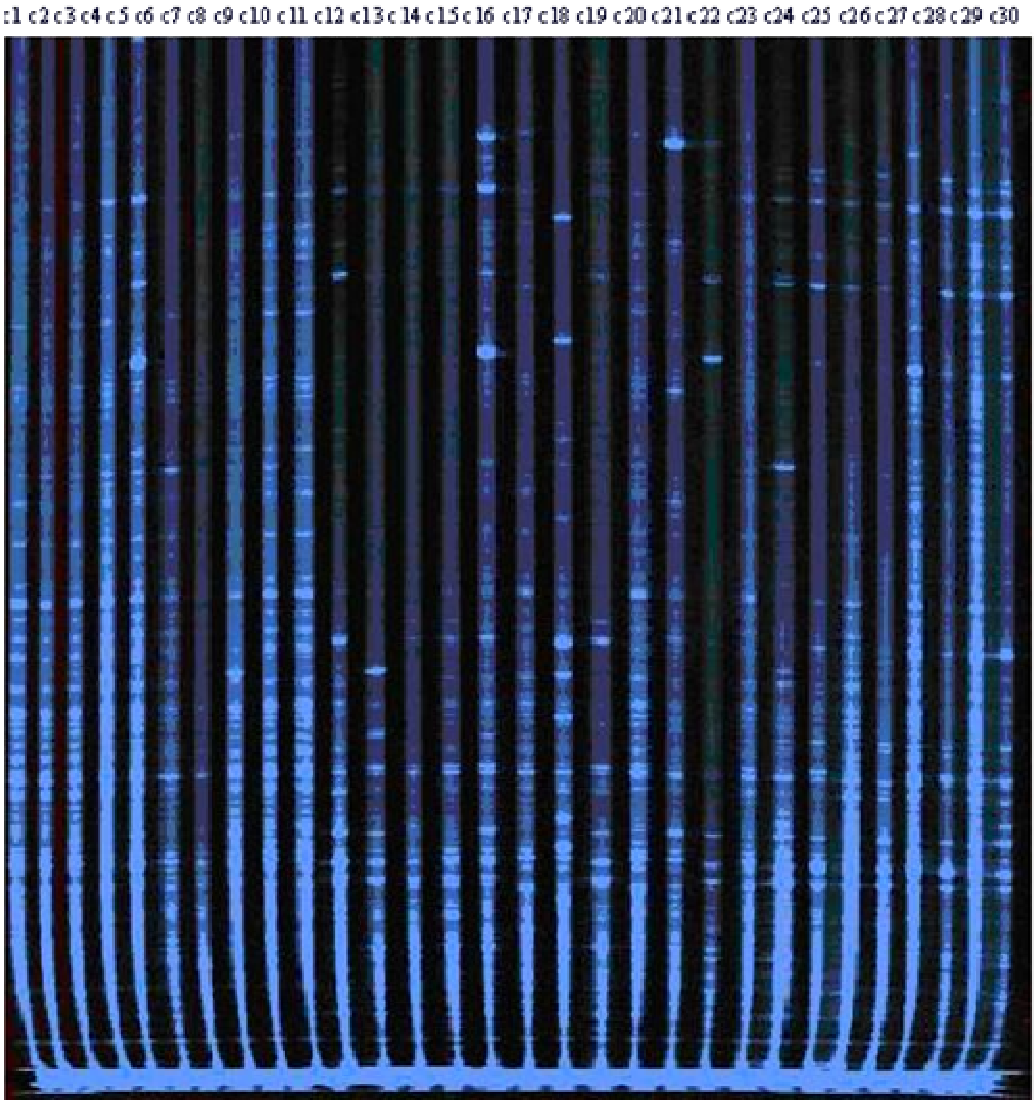

Figure 1. AFLP fingerprints among 30 *Ganoderma* strains. The DNA fingerprints were generated using the AFLP Primer *EcoR* I -ACA/ *Mse* I -CTC. c1-c30 lanes: 1-30 strains.

*Mse*I-CTC (Figure 1).

Phylogenetic analysis

In AFLP, all the strains were clustered into eight groups (Figure 2). Genetic analyses of six strains are from Agricultural Culture Collection of China in AFLP. Strains 8, 9, 10, 11, 12, 30 were divided into two groups, strains 8, 9, 10 had the same polymorphic bands, were clustered to sect. *Phaeonema*; and strains 11, 12, 30 were clustered to sect. *Ganoderma*. The results are identical to classical taxonomy and molecular taxonomy (Zhao and Zhang, 2000); all these six strains are divided into sect. *Phaeonema* and sect. *Ganoderma*. According to this standard, 30 strains were divided into eight groups. Most of strains fell into group 1, and belonged to sect. *Phaeonema*. Strains of groups 2, 3, 4, 5, and 6 belonged to sect. Strains of groups 7 and 8 had high genetic diversity, and their taxonomic position located above sect. *Phaeonema* and sect. *Ganoderma*. Group 1 had 14 strains as follows: strains 1, 2, 3, 4, 5, 6, 8, 9, 10, 16, 19, 22, 27 and 29, group 5 had 9 strains, which was composed of strains 11, 12, 14, 15, 23, 24, 26, 28 and 30, while group 6 included strains 13 and 18. Strains 7, 20 and 25 belonged to group 2, 3 and 4, respectively.

DISCUSSION

Ganoderma spp. are traditional medicinal mushroom in China, and Ling Zhi was divided into six types by different colors, such as Qing Zhi (Cyan), Chi Zhi (Red, *G. lucidium*), Huang Zhi (Yellow), Bai Zhi (White), Hei Zhi

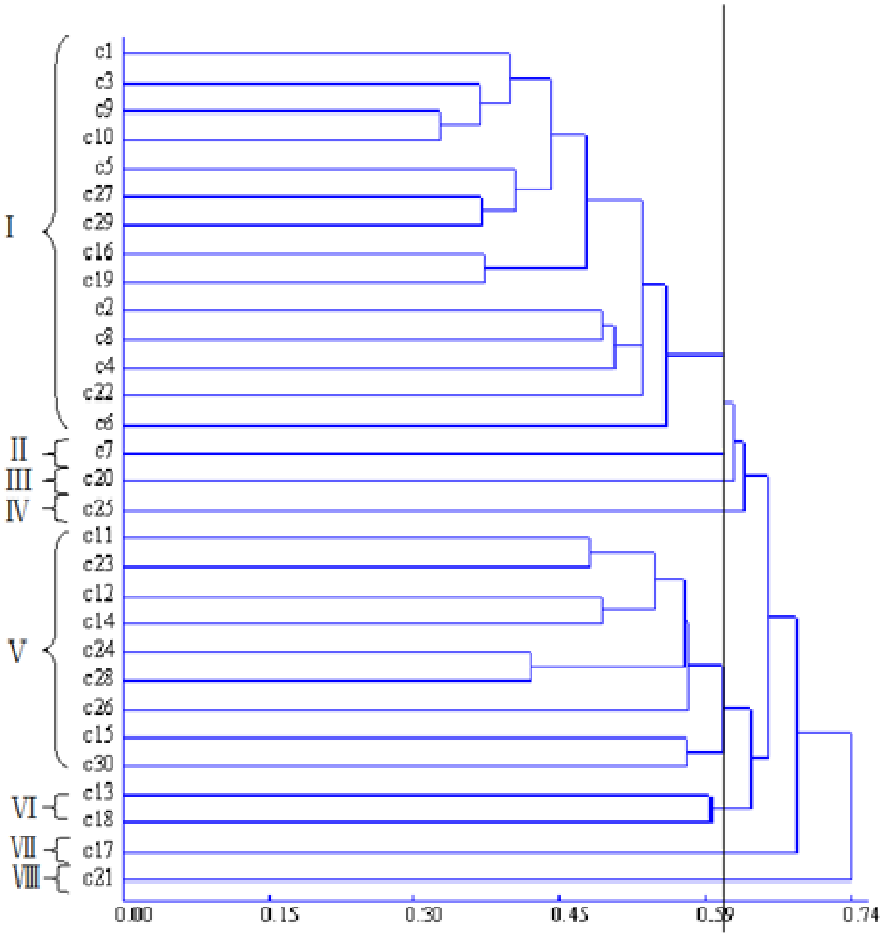

Figure 2. UPGMA dendrogram based on the dissimilarity of AFLP patterns.

(Black), and Zi Zhi (Purple, *G. sinensin*) in the ancient Chinese medical encyclopedias (Lin, 2001). Indeed, *Ganoderma* is a general term of Ganodermataceae (Chen and Li, 2004). 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).

There were three kinds of *Ganoderma*, which are *G. lucidum*, *G. sinense* and *G. tsugae* can be used as medical and edible mushroom, regarded in "Can be used as health food list of fungal species", which was released by Chinese Ministry of Health in 2001 (Chinese Pharmacopoeia Committee, 2005; Zhou et al., 2006). Currently, the amount of demand about *G. lucidum* is very larger in the world. In 1997, the *G. lucidum* productions are 4300 tons in the world, of which China accounted for 3000 tons. In 1999, it has been reported that the annual production value of *G. lucidum* products in the world is 1628.4 million U.S. dollars, in China for 350 million U.S. dollars, and in 2000, the annual production values of *G. lucidum* products are more than 2 billion U.S. dollars (Xiao et al., 2006).

With the increase of the production and sales, people attach great importance to its cultivation, breeding, preservation processing etc. *Ganoderma* breed is the most important basic means of production, which is the prerequisite and key of the *Ganoderma*'s production development. The breed's quality determines its production and quality. Identifying the species of *Ganoderma* and establishing its DNA fingerprinting will be helpful for exact strain identification from the perspective of molecular biology. Thus we can effectively utilize the advantages of different species of *Ganoderma*, further develop its cultivation value and promote the development of production. A variety of molecular marking methods have been used in the identification of cultivation of *Ganoderma,* such as RAPD (Zhao et al., 2003; Luo et al., 2005), ITS PCR-RFLP (Luo et al., 2005; Su et al., 2007), AFLP (Zheng et al., 2007). PCR-RFLP has characteristics of good stability and high repeatability, which is used in phylogenetic study of microbiology. Its resolution is high in genera or genus, but the intraspecific polymorphism is not satisfying. Research also shows that it has good resolution in different species, but it cannot reveal the difference bet-

ween strains. Having the characteristics of large quantity and high sensitivity, RAPD takes the whole genome as a target, and is suitable for the identification of different species. But its stability is not satisfying. Based on the difference of strain's fingerprint, AFLP can effectively distinguish the genotypes of different strains, which could provide effective technical means for quality control of cultivated strains and strains identification. AFLP has been successfully used in identification of main cultivated species of shiitake. PCR-RFLP has characteristics of good stability and high repeatability, which is used in phylogenetic study of microbiology. Its identification could accurate to genera or genus, but intraspecific polymorphism is not satisfying. Research also shows that it has good identification in different species, but it cannot reveal the difference between strains. Having the characteristics of large quantity and high sensitivity, RAPD takes the whole genome as a target, and is suitable for the identification of different species. But its stability is not satisfying. Based on the difference of strain's fingerprint, AFLP can effectively distinguish the genotypes of different strains, which could provide effective technical means for quality control of cultivated strains and strains identification. AFLP has been successfully used in identification of main cultivated species of shiitake (Zhuo et al., 2006).

In the study, six strains were chosen from Agricultural Culture Collection of China as control, in order to identify 24 other strains that were not ascertained in taxonomic position and medical value. All six controls were clustered to two groups, sect. Phaeonema and sect. Ganoderma. Compared with traditional taxonomical system, these clustering results seem to be in agreement with the taxonomical system. In AFLP, six strains collected from Microbiological culture and Collection Center of Guangdong Institute of Microbiology were similar with sect. Phaeonema, and were clustered to group 1. The result indicated that they belonged to sect. Phaeonema, and had the equal medical value to strains of sect. Phaeonema. Strains 7, 13, 24 and 28 named Ganoderma (Korea) were clustered to sect. Ganoderma, though they were collected from different sites. In four strains, strains 24 and 28 had high similarity coefficient, but strains 7 and 13 had low similarity coefficient. Strain 21 was less similar to these four strains, though it still belonged to sect. Ganoderma. It was deduced that Ganoderma (Korea) had some variation after introduced from original sites. These variances are related to different ecological habitats, and caused subtle discrimination in morphological traits and medical values. The data obtained in this study demonstrated that AFLP as a tool to identify the Ganoderma species or cultivars is very sensitive and practical.

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