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Development of RAPD-SCAR markers for different *Ganoderma* species authentication by improved RAPD amplification and molecular cloning

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ABSTRACT. The sequence-characterized amplified region (SCAR) is a valuable molecular technique for the genetic identification of any species. This method is mainly derived from the molecular cloning of the amplified DNA fragments achieved from the random amplified polymorphic DNA (RAPD). In this study, we collected DNA from 10 species of *Ganoderma* mushroom and amplified the DNA using an improved RAPD technique. The amplified fragments were then cloned into a T-vector, and positive clones were screened, indentified, and sequenced for the development of SCAR markers. After designing PCR primers and optimizing PCR conditions, 4 SCAR markers, named LZ1-4, LZ2-2, LZ8-2, and LZ9-15, were developed, which were specific to *Ganoderma gibbosum* (LZ1-4 and LZ8-2), *Ganoderma sinense* (LZ2-2 and LZ8-2), *Ganoderma tropicum* (LZ8-2), and *Ganoderma lucidum* HG (LZ9-15). These 4 novel SCAR markers were deposited into GenBank with the accession Nos. KM391935, KM391936, KM391937,

and KM391938, respectively. Thus, in this study we developed specific SCAR markers for the identification and authentication of different *Ganoderma* species.

Key words: Genetic identification; Random amplified polymorphic DNA; *Ganoderma* species; Sequence-characterized amplified region

INTRODUCTION

In East Asian countries, particularly China and Japan, mushrooms of *Ganoderma* species have been cultivated and used as traditional medicines for thousands of years. They are commonly known as 'Reishi' in Japan or as 'língzhī' in China. *Ganoderma* mushrooms are popular because of their therapeutic potential against several life-threatening diseases. Among different species, *Ganoderma lucidum* is the most popular and well explored for its beneficial health activities (Paterson, 2006; Sanodiya et al., 2009; Mahajna et al., 2009; Mei et al., 2014a). Other species have gained less attention; however, they also have significant medicinal values. For example, *Ganoderma sinense* has immunomodulatory, anticancer, and antiviral activities (Liu et al., 2009; Sato et al., 2009; Yue et al., 2013), while *Ganoderma tropicum* has acetylcholinesterase inhibitory activity and neuro-beneficial activity (Hu et al., 2013). *Ganoderma gibbosum* is also used as a medicinal fungus in China (Chen et al., 2010). These *Ganoderma* mushrooms possess numerous variations in the size, shape, and color, and it is often difficult to identify and morphologically distinguish them. Thus, genetic characterization and identification are important for *Ganoderma* mushrooms.

Random amplified polymorphic DNA (RAPD), inter-simple sequence repeat, simple sequence repeat, and amplified fragment length polymorphism analyses are the major molecular marker technologies currently used for the genetic characterization and identification of an organism (Williams et al., 1990; Agarwal et al., 2008; Fu et al., 2013; Noormohammadi et al., 2013; Mei et al., 2014a,b). The sequence characterized amplified region (SCAR) marker is another molecular marker, which is more stable and is generally derived from RAPD or inter-simple sequence repeat (Dnyaneshwar et al., 2006; Su et al., 2007; Li et al., 2010; Kumla et al., 2012; Rajesh et al., 2013). When SCAR with RAPD are combined, molecular analysis is simplified, in which PCR primers are designed from the sequence of the RAPD amplicon to develop SCAR markers (Kumla et al., 2012; Rajesh et al., 2013).

We previously used an improved RAPD technique for the genetic characterization of different *Ganoderma* species (Mei et al., 21014a). In this study, we developed 4 SCAR markers after the molecular cloning of RAPD fragments obtained from the DNA materials of different *Ganoderma* species. These markers were found to be specific to certain *Ganoderma* species.

MATERIAL AND METHODS

Extraction of DNA from *Ganoderma* species

DNA was extracted from different samples of *Ganoderma* species (Table 1) and other samples by using standard methods. DNA samples were then diluted to a final concentration of 10 ng/μL and stored at -20°C until use (Fu et al., 2013).

Table 1. Sources of *Ganoderma* samples for RAPD-SCAR.

No.	Accession name	Sources of *Ganoderma*	Deposit No.
1	*Ganoderma gibbosum* (Blumii et Nees) Patouillard	Guangdong Culture Collection Center	GIM5.6
2	*Ganoderma tropicum* (Jungh.) Bres.	Guangdong Culture Collection Center	GIM5.289
3	*Ganoderma applanatum* (Pers.ex Wullr) Pat	Guangdong Culture Collection Center	GIM5.282
4	*Ganoderma australe* (Fr.) Pat	Guangdong Culture Collection Center	GIM5.288
5	*Ganoderma sinense*	Inst. Microbiology of Chinese Academy of Sciences	CGMCC5.0069
6	*Ganoderma lucidum* HG	Inst. Edible Fungi of Fujian academy of Agricultural Sciences	ACCC51329
7	*Ganoderma lucidum* (Curtis) P. Karst	Inst. Edible Fungi of Fujian Academy of Agricultural Sciences	CFCC85862
8	*Ganoderma lucidium*	Inst. Microbiology of Chinese Academy of Sciences	CGMCC5.0026
9	*Ganoderma neojaponicum* Imazeki	Beijing Agricultural University	CFCC87599
10	*Ganoderma lucidium* (Leysser Fr.) Karst.	Inst. Wensheng Edible Fungi in Shantou	GIM5.250SL

Amplification and recovery of improved RAPD fragments

The improved RAPDs were amplified by polymerase chain reaction (PCR) using the random primers SBC-M7 and SBC-I1 DNA from 10 *Ganoderma* species (Table 1). The 15 μL PCR mixture consisted of 7.5 μL 2X Taq PCR MasterMix, 1.5 μL 2.5 μM primer, 1.5 μL genomic DNA, and ddH_2O. Amplification reactions were performed using the machine Veriti® 96-Well Thermal Cycler" (Applied Biosystems, Foster City, CA, USA), with the following steps: initial denaturation at 95°C for 90 s, 40 cycles of denaturation at 94°C for 40 s, annealing at 36°C for 60 s, with the annealing to extension temperature increasing at 0.125°C/s (5% ramp rate), extension at 72°C for 90 s, and a final extension step at 72°C for 5 min. PCR products were separated on a 1.5% agarose gel by electrophoresis for 40 min. The bright bands were excised from the agarose gel, purified using the TIANgel Mini Purification Kit (DP209, Tiangen Biotech, Beijing, China) according to the manufacturer protocol.

Cloning, screening, and sequencing of DNA fragments

The purified DNA fragments were ligated into the pGM-T vector (No. VT202, Tiangen) and transformed into DH5α *Escherichia coli* competent cells. The recombinant clones were selected on Luria Bertani (LB) agar plates containing 100 μg/μL ampicillin, 40 mg 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside, and 160 μg isopropyl β-D-1-thiogalactopyranoside. The white colonies were screened out by blue/white screening. The presence of the correct insert was verified by PCR using the T7/SP6 primer pairs (Yang et al., 2013), located in the pGM-T vector near the ligation ends, and *Eco*RI digestion (Fu, 2012). The cloned DNA fragments were then sequenced using the Sanger method.

Homological analysis and SCAR primer design

The homology of sequenced DNA was searched and analyzed using the online program BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) in different species. The nucleotide sequence of each cloned RAPD fragment was used to design pairs of SCAR primers using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). The sequences of each primer are listed in Table 2.

Development SCAR markers and SCAR analysis

To develop SCAR markers, PCR amplification was performed. The 10 μL PCR system

Table 2. Sequences of SCAR primers, PCR condition and product size.

SCAR	5'-primer	Sequence (5'-3')	3'-primer	Sequence (5'-3')	Size (bp)	Tm (°C)
LZ1-4	LZ1-4L	GTGTTTCTGGCATGCACACC	LZ1-4R	ACACAGTACTTCACCGACGG	211	62
LZ2-2	LZ2-2L	TGAGGATTGGAAACGGGGTG	LZ2-2R	CTCTGGTGTTTGGATTGCGC	272	62
LZ8-2	LZ8-2L	AACCGCCAAGACACTGTAGG	LZ8-2R	CTCTCATCGGGTTCACTCGG	291	60
LZ9-15	LZ9-15L	ACCACCTACCTGCTCCTCTT	LZ9-15R	TCCTTCCGGCAGTGGTAGTA	262	60

included: 5 μL 2X Taq PCR MasterMix, 1 μL 2.5 μM of each SCAR primer, 1 μL 10 ng genomic DNA, and 3 μL ddH_2O. PCR was performed by using the Veriti® 96-Well Thermal Cycler (Applied Biosystems) with an initial pre-denaturation for 90 s at 95°C, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at temperatures 60° or 62°C for 30 s, and extension at 72°C for 40 s. The final extension step was performed at 72°C for 5 min. Sequences of the 4 pairs of SCAR primers, amplified length, and PCR conditions are listed in Table 2. To identify differences between the varieties of *Ganoderma* sp and other species of medicinal plants, SCAR analysis was performed using 23 DNA samples as templates, including 10 samples of *Ganoderma* sp, which were described previously (Mei et al., 2014a), 2 samples of *Dimocarpus longan* collected from Fujian and Hainan (Yang et al., 2013), 2 samples of *Lonicera japonica* collected from Guangdong and Hubei (Fu et al., 2013), 1 *Gardenia jasminoides*, 1 *Litchi chinensis* from Guangdong, 1 *Dimocarpus confinis* from Guangxi, 1 *Canarium album* from Luzhou City, *Gastrodia elata* collected from Liangshan City in Sichuan Province, 1 *Penthorum. sedoides*, *Penthorum chinense* collected from Gulin County in Sichuan Province, and *Violoa philippica* (Yang et al., 2013), and 1 *Angelica sinensis* from Sichuan Province. The amplified PCR products were separated by electrophoresis on a 1.0% agarose gel in 1X TAE buffer at 120 V for 40 min. Gels were visualized by 0.5 μg/mL ethidium bromide staining, and the images were documented using the ChemiDoc XRS (Bio-Rad, Hercules, CA, USA).

RESULTS

Recovery of RAPD fragments

Two RAPD primers, SBC-M7 and SBC-I1, were used to improve RAPD amplification from DNA samples of *Ganoderma* species (Mei et al., 2014a). The results are shown in Figure 1A; the blue arrows indicate bands that were cut from the agarose gel and labeled with L1, L2, L8, and L9, respectively. DNA from the agarose gel was purified using the TIANgel Mini Purification Kit and eluted with 20 μL ddH_2O. To check DNA quality and measure the quantity for ligation, 2 μL purified PCR products and 0.5 μL T-vector was added to each well of an agarose gel. Figure 1B shows the DNA quality and quantity.

Cloning of RAPD fragments

Based on DNA quality, an appropriate amount of PCR product and T-vector were ligated together and screened using the blue/white method. Positive clones were then identified by PCR amplification using SP6/T7 primers (Figure 2). The plasmids from positive clones in Figure 2 in red were further extracted and cut by *Eco*RI enzyme digestion (Figure 3). In lane 2 of Figure 3, clone LZ1-4, which showed a 550-base pair (bp) DNA fragment, was sequenced. In lane 4 of Figure 3, clone LZ2-2, which showed a 600-700-bp inserted DNA fragment, was

sequenced. In lane 6 of Figure 3, clone LZ8-2, which showed a 550-650-bp inserted DNA fragment, was sequenced. In lane 8 of Figure 3, clone LZ9-15, which showed a 550-650-bp inserted DNA-fragment, was sequenced.

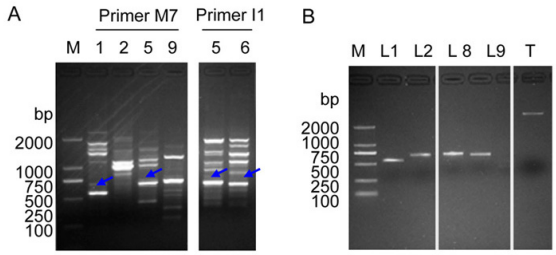

Figure 1. Amplification, quality and quantity checking of improved RAPD fragments. **A.** Improve RAPD amplification from DNA samples of *Ganoderma* species. *Lanes 1*, *2*, *5*, *6*, and *9* = different *Ganoderma* species samples listed in Table 1. The blue arrows indicate bands before cut. **B.** Quality and quantity checking of improved RAPD fragments purified from agarose. *Lanes L1*, *L2*, *L8*, and *L9* indicate the PCR fragments LZ1, LZ2, LZ8, and LZ9, respectively. "T" is T vector. *Lane M* indicates the DNA molecular weight marker DL2000 with the fragment size of 2000, 1000, 750, 500, 250, 100 bp.

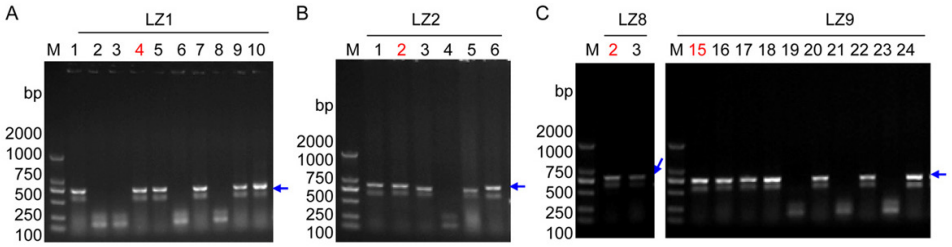

Figure 2. DNA cloning and identification of positive clones. **A.** Clone identification of RAPD fragment LZ1. *Lanes 1-10* indicate different clones. **B.** Clone identification of RAPD fragment LZ2. *Lanes 1-6* indicate different clones. **C.** Clone identification of RAPD fragment LZ8. *Lanes 2-3* indicate different clones. Clone identification of RAPD fragment LZ9. *Lanes 15-24* indicate different clones. The blue arrows indicate positive PCR products. Clones LZ1-4, LZ2-2, LZ8-2 and LZ9-15 in red colors were picked up to extract the plasmid DNA. The blue arrows indicate expected PCR bands in size of different clones. *Lane M* indicates the DNA molecular weight marker DL2000 with the fragment size of 2000, 1000, 750, 500, 250, 100 bp.

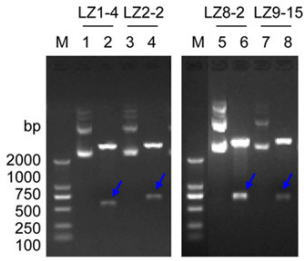

Figure 3. Identification of positive clones by plasmid DNA digestion. *Lanes 1* and *2* indicate clone LZ1-4 plasmid DNA without or with *Eco*RI digestion. *Lanes 3* and *4* indicate clone LZ2-2 plasmid DNA without or with *Eco*RI digestion. *Lanes 5* and *6* indicate clone LZ8-2 plasmid DNA without or with *Eco*RI digestion. *Lanes 7* and *8* indicate clone LZ9-15 plasmid DNA without or with *Eco*RI digestion. The blue arrows indicate expected inserts of RAPD DNA fragments.

Sequences and characterization of *Ganoderma* species-specific RAPD fragments

Sequencing of the above 4 cloned RAPD fragments revealed that clone LZ1-4 consisted of 555 nucleotides and was deposited into GenBank with accession No. KM391935 (Figure 4A), clone LZ2-2 consisted of 658 nucleotides and was deposited into GenBank with accession No. KM391936 (Figure 4B), clone LZ8-2 consisted of 598 nucleotides and was deposited into GenBank with accession No. KM391937 (Figure 4C), and clone LZ9-15 consisted of 567 nucleotides and was deposited into GenBank with accession No. KM391938 (Figure 4B). BLAST searches of the nucleotide sequences in GenBank showed that no cloned DNAs showed identity to other species (data not shown).

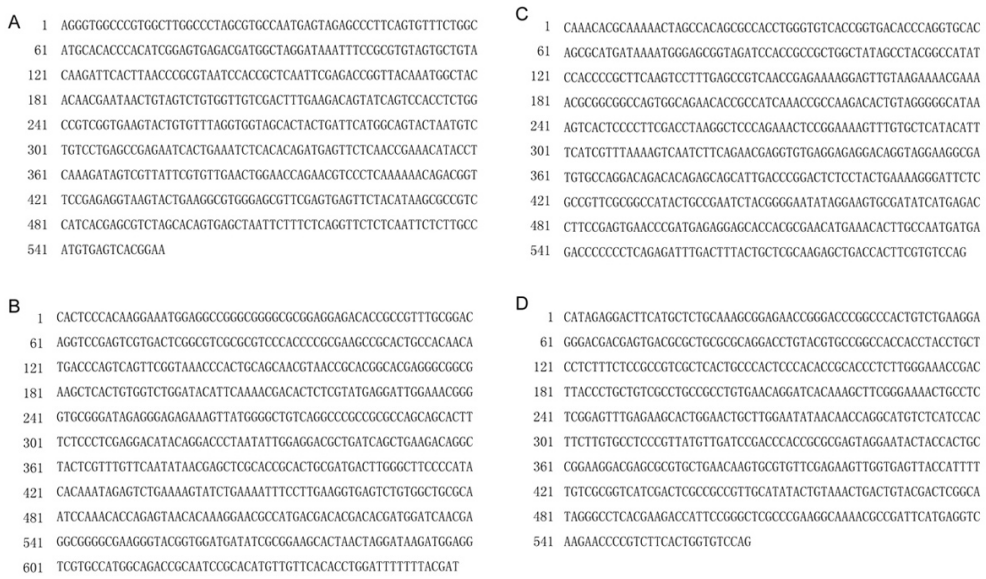

Figure 4. Cloned sequences information by Sanger-sequencing. **A.** Sequences of clone LZ1-4. **B.** Sequences of clone LZ2-2. **C.** Sequences of clone LZ8-2. **D.** Sequences of clone LZ9-15.

Development of *Ganoderma* species-specific SCAR markers

To generate stable diagnostic *Ganoderma* species-specific SCAR markers from RAPD markers, 4 pairs of primers (Table 2) were designed and synthesized based on cloned sequences. The designed SCAR primer pairs were then used to amplify genomic DNA from 23 of collected DNA samples to test amplification species-specificity. The results are shown in Figure 5, the SCAR maker LZ1-4 showed an expected size in a sample *G. gibbosum* (Blumii et Nees) Patouillard of 10 *Ganoderma* species (lane 1 of Figure 5A), without any amplification in other species tested; the SCAR maker LZ2-2 showed an expected size in a *Ganoderma sinense* sample of 10 *Ganoderma* species (in lane 5 of Figure 5B), without any amplification in other species tested; the SCAR marker LZ8-2 showed an expected size in a *G. sinense* sample of 10 *Ganoderma* species (lane 5 of Figure 5C) and 2 weak bands in the sample of *G. gibbosum* (Blumii et Nees) Patouillard (lane 1 of Figure 5C), and sample of *G. tropicum* (Jungh.) Bres (lane 2 of Figure 5C), without any amplification in other species tested; the SCAR maker

LZ9-15 showed an expected size in an *G. lucidum* HG sample of 10 *Ganoderma* species (lane 6 of Figure 5D), without amplification in other species tested. These results indicate that these SCAR markers were sample-specific, not species-specific. The lack of this specific amplicon in the intra-species or other species indicates the efficacy of this marker for distinguishing the *Ganoderma* samples.

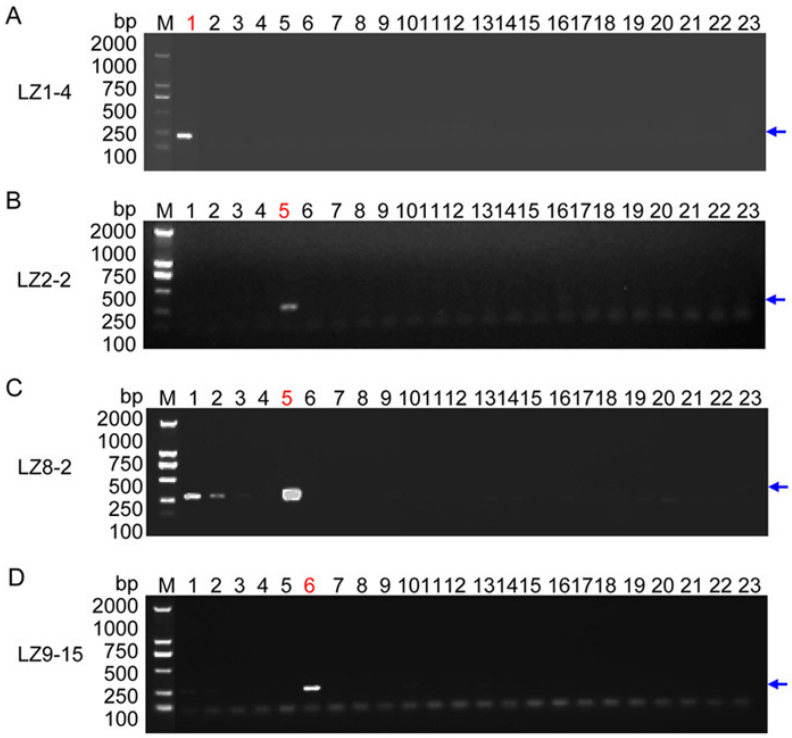

Figure 5. Development of RAPD-SCAR markers for LZ1-4, LZ2-2, LZ8-2 and LZ9-15. **A.** A SCAR marker LZ1-4. **B.** A SCAR marker LZ2-2. **C.** A SCAR marker LZ8-2. **D.** A SCAR marker LZ9-15. *Lanes 1-10* indicate the different *Ganoderma* species samples listed in Table 1. *Lanes 11-23* are DNA samples prescribed in Material and Methods. *Lanes 11* and *12* are two samples of *Dimocarpus longan*; *Lanes 13* and *14* are two samples of *Lonicera japonica*; *lane 15* is one sample of *Gardenia jasminoides*; *lane 16* is one sample of *Litchi chinesis*; *lane 17* is one sample of *Dimocarpus confinis*; *lane 18* is one sample of *Canarium album*; *lane 19* is one sample of *Gastrodia elata*; *lane 20* is one sample of *Penthorum sedoides*; *lane 21* is one sample of *Penthorum chinense*; *lane 22* is one sample of *Violoa philippica*; *lane 22* is one sample of *Angelica sinensis*. The red arrows indicate expected PCR products in size. *Lane M* indicates the DNA molecular weight marker DL2000.

DISCUSSION

Mushrooms in the *Ganoderma* genus are consumed as a source of natural medicine and are known for their health benefits and therapeutic activities. Some species of the *Ganoderma* mushrooms are more effective than others in terms of edibility and medicinal usage. Identification of specific *Ganoderma* species would be helpful for consumers, health professionals, and systemic mycobiologists. In this study, we developed SCAR molecular markers for the identification of 3 species of *Ganoderma* genus based on previous RAPD genetic char-

acterization (Mei et al., 2014a).

Molecular marker technology, particularly the RAPD technique, and improved RAPD analysis has become a common and efficient tool for molecular analysis or genetic characterization of different species. A combination of SCAR and RAPD has significantly improved the stability and authenticity of the technique (Dnyaneshwar et al., 2006; Su et al., 2007; Li et al., 2010; Kumla et al., 2012; Rajesh et al., 2013; Fu et al., 2013; Noormohammadi et al., 2013; Mei et al., 2014a,b). Previous studies successfully developed RAPD markers for different species of animals, plants, and microbes (Lee et al., 2013; Yang et al., 2013, 2014; Dutta et al., 2014). However, no SCAR marker for the identification of different *Ganoderma* species has been reported. In this study, we developed 4 SCAR markers for the identification of different species, including *G. gibbosum*, *G. sinense*, *G. tropicum*, and *G. lucidum* HG.

The 555-nucleotide SCAR marker LZ1-4 was established to identify or authenticate *G. gibbosum*, which has been deposited into GenBank with accession No. KM391935, while LZ2-2 of 658 nucleotides has been deposited into GenBank with accession No. KM391936 specific to *G. sinense*. The LZ8-2 marker of 598 nucleotides was established to identify *G. sinense*; however, this marker also possesses a weaker level of specificity to *G. gibbosum* and *G. tropicum*. The GenBank accession No. for LZ8-2 is KM391937. Another SCAR marker, LZ9-15, was found to be specific to *G. lucidum* HG. This marker consists of 567 nucleotides, and has been deposited in to GenBank with the accession No. KM391938. Because BLAST searches of the nucleotide sequences in GenBank revealed no matches to these sequences, these novel markers can be used for the identification of these 4 species of *Ganoderma* mushroom. Typically, the different species of *Ganoderma* mushrooms vary in morphology and chemical constituents. *G. lucidium* (Leysser Fr.) Karst and *G. lucidium* HG are relatively well-known for their nutritional and medicinal importance compared to other species, such as *G. gibbosum*, *G. tropicum*, and *G. sinense*, while *G. lucidum* is bitter in taste compared to *G. sinense*. The variable medicinal importance may be because different *Ganoderma* species have different genetic characteristics and their genetic materials are highly polymorphic. The diverse range of genetic characteristics of *Ganoderma* mushrooms also influences their growth conditions. For example, *G. lucidum* HG is typically grown in Northern and North-Eastern China in cold environments, while *G. tropicum* is grown in tropical forests. A sequence-related amplified polymorphism analysis by Sun et al. (2006) revealed significant genetic variation between *G. lucidum* and *G. sinense*. Zhao et al. (2003) showed 80-100% polymorphism in genetic materials in different *Ganoderma* species, and Mei et al. (2014b) showed high levels of genetic distance between *G. gibbosum*, *G. tropicum*, *G. sinense*, and *G. lucidum*. This high genetic variability enabled the generation of species-specific SCAR markers. These molecular markers may have roles in ecological preservation, molecular identification, and authentication, as well as genetic characterization of these medicinal mushrooms.

Conflicts of interest

The authors declare no conflict of interest.

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