2002

Comparative analysis of the ITS rDNA sequence and nutrient compositions of an un-named Ganoderma species in Australia

Li-Xia Liu
University of Wollongong

Peter Howe
University of Wollongong

Chen-Wei Su
University of Wollongong

Fei Sun
Jilin University

Ren Zhang
University of Wollongong

Comparative analysis of the ITS rDNA sequence and nutrient compositions of an un-named Ganoderma species in Australia

Abstract

An Australian species of Ganoderma genus (temporally named Ganoderma nt) mistaken for Garoderma lucidum, a well-known herbal medicine, was examined with internal transcribed spacer ribosomal DNA (ITS rDNA) sequence as an aid to the taxonomy. Variation between G. nt and G. lucidum in the ITS rDNA sequence was 2% - 4 %. Also nutrient value in this species was analyzed compared with G. lucidum. G. nt had similar contents to G. lucidum in polysaccharides and monosaccharides on dry mass base in fruit body. However, G. nt fruit body had higher soluble protein (14 mg/g dry mass) and fatty acids (5.6 mg/g dry mass) contents. Mycelia of G. nt obtained from shaking and static cultures had higher contents of polysaccharides and monosaccharides (100 - 140 mg/g dry mass) than fruit bodies (~13 mg/g dry mass). These results indicate that G. nt is closely related to G. lucidum in morphology (lower genetic variability; taxonomy) and nutrient value, and is likely to have comparable function as a herbal medicine or dietary supplementation.

Disciplines

Arts and Humanities | Life Sciences | Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Liu, L., Howe, P., Su, C., Sun, F. & Zhang, R. (2002). Comparative analysis of the ITS rDNA sequence and nutrient compositions of an unnamed Ganoderma species in Australia. Ganoderma: genetics, chemistry, pharmacology and therapeutics. Proceedings of the International Symposium on Ganoderma Research, 21-23 October 2002 (pp. 67-74). Beijing, China: Beijing Medical University Press.

Comparative Analysis of the ITS rDNA Sequence and Nutrient Compositions of an Unnamed *Ganoderma* Species in Australia

Li-Xia Liu[1,2] Peter Howe[1] Chen-Wei Su[1] Fei Sun[3] Ren Zhang[1]

[1] *Smart Foods Centre and Department of Biological Sciences, University of Wollongong, Wollongong, NSW 2522 Australia*
[2] *Life School, North-East Normal University, Changchun, China*
[3] *Institute of Cell Biology, Jilin University, Changchun, China*

ABSTRACT

An Australian species of *Ganoderma* genus (temporally named *Ganoderma nt*) mistaken for *Ganoderma lucidum*, a well-known herbal medicine, was examined with internal transcribed spacer ribosomal DNA (ITS rDNA) sequence as an aid to the taxonomy. Variation between *G. nt* and *G. lucidum* in the ITS rDNA sequence was 2% ~ 4%. Also nutrient value in this species was analyzed compared with *G. lucidum*. *G. nt* had similar contents to *G. lucidum* in polysaccharides and monosaccharides on dry mass base in fruit body. However, *G. nt* fruit body had higher soluble protein (14 mg/g dry mass) and fatty acids (5.6 mg/g dry mass) contents. Mycelia of *G. nt* obtained from shaking and static cultures had higher contents of polysaccharides and monosaccharides (100 ~ 140 mg/g dry mass) than fruit bodies (~ 13 mg/g dry mass). These results indicate that *G. nt* is closely related to *G. lucidum* in morphology (lower genetic variability; taxonomy) and nutrient value, and is likely to have comparable function as a herbal medicine or dietary supplementation.

Key words: *Ganoderma lucidum*, internal transcribed spacer ribosomal DNA, compositions

INTRODUCTION

Edible fungi have been valued throughout the world as both food and medicine for thousands of years. Certain species of *Ganoderma*, a genus of fungi, were originally used as herbal medicine in China, called Lingzhi (Reizhi in Japanese), *Ganoderma lucidum*. They have become accepted as an important source of the health-maintaining nutriceuticals, especially in the Pan-Pacific regions (Brower, 1988; Chang, 1999; Zeisel, 1999; Wasser et al. 2000). Lingzhi is unique with a wide range of health-giving properties: it strengthens the immune system and has general prophylactic effects. The major active ingredients in *Ganoderma* are polysaccharides, triterpenes, immunomodulatory proteins, organic germanium and vitamins (Mizuno T, 1995; Lin ZB, 1996). *Ganoderma*-based products have attracted a great deal of attention during the last decade and the market value of these products in 1995 alone has been estimated to exceed US $ 1.6 billion (Chang and Buswell, 1999). Imported *Ganoderma*-products have recently become available in Australia and are consumed mainly by Asian communities.

More than 250 *Ganoderma* species have been recorded worldwide (Chang, 1999). Many of these are found all over Australia, particularly in tropical rainforest and temperate areas (Smith and Sivasithamparam, 2000). There have been no attempts as yet to assess these fungi for their food or medicinal value, even though some of the popular species, such as *G. applanatum* and *G. australe*, being used for Lingzhi are available locally (Yeh and Chen, 1990).

In this paper we compared the sequences of the internal transcribed spacer ribosomal DNA (ITS rDNA) of an Australian *Ganoderma* species which is an unnamed species and mistaken for *G. lucidum* which is very common in Asian countries (Cooke, 1883; Blackford 1944) or named *Ganoderma* sp. aff.

lucidum (Hood et al., 1996). Smith and Sivasithamparam (2000) considered this species would be a new species, most likely arising from vicariance species for *G. lucidum*. Also the nutrient composition including polysaccharides, soluble protein and fatty acids of this unnamed *Ganoderma* species was examined.

MATERIALS AND METHODS

Materials collection

The unnamed Australian *Ganoderma* species was collected in Darwin, NT of Australia (provided by Ms Lanni Zhang; and named *Ganoderma nt* temporarily) (Figure. 1). An imported *G. lucidum* strain from China obtained from a pharmacy shop in Sydney.

Figure 1. The fruit body of *Ganoderma nt* (Darwin NT, Australia).

Mycelia culture

Small pieces of tissues separated from fresh fruit body of *G. nt* were maintained on Potato Dextrose Agar (PDA) medium at 28℃ for 7 days to get mycelia (Figure 2a) (PDA medium is a common medium used in mushroom culture). Mycelia culture in a liquid medium was incubated in a flask in an orbital incubator shaker operated at 120 rpm or in a petric dish in a static incubator, containing 0.2% yeast extract, 1% bacto peptone, 2% glucose, 0.05% $MgSO_4$ and 0.1% KH_2PO_3 at 28℃ for 6 days. In the shaking culture, mycelia formed a small ball with 0.3 ~ 0.8cm diameter depending on the shaking speed and temperature (Figure 2b).

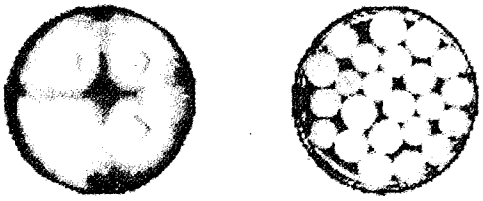

Figure 2. *Ganoderma nt* mycelium cultured in a PDA medium (a) and in a liquid medium on shaking culture (b).

rDNA analysis and PCR amplification

G. nt DNA was extracted from approximately 2 g of wet cultured mycelia which was ground in a mortar and pestle with sand. 4 ml of lysis buffer was gradually added during the grinding, after which 750 μl aliquots were transferred to microcentrifuge tubes. The samples were digested at 65℃ for 30 minutes before extraction with equivalent amounts of phenol : chloroform : isoamyl alcohol (50:48:2). The samples were then centrifuged (15 min, 7000g) in a bench microcentrifuge, after which the upper aqueous layer (DNA containing layer) was transferred into new microcentrifuge tubes. Sodium acetate (3M, 1/10^{th} vol.) and 80% ethanol (2.5× vol.) were added to the sample and the DNA from each microcentrifuge tube was pooled and washed three times with 75% ethanol, with the DNA being aspirated dry between washes. The DNA was then dissolved in minimal water with gentle heating at 37℃.

The primers used were either ITS-1 (5′-TACCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) or BMB-CR (5′-GTACACACCGCCCGTCG-3′) and LR-I (5′-GGTTGGTTTCTTTTCCT-3′) from Smith and Sivasithamparam (2000). The PCR was performed with an Applied Biosystems GeneAmp 9600. The reaction mix for each PCR amplification consisted of 5 μl PCR master mix, 1 μl of each primer, 1 or 2 μl of DNA template (~ 10 ng/μl), and nuclease-free water to a total of 10 μl. The time programme used for the amplification consisted of a preheating step (95℃ for 1 min) followed by 28 cycles of 94℃, 1 min→50℃, 0.5 min → 70℃, 1 min. The samples were then held at 4℃ to stop further PCR.

DNA sequencing from both the 5′-and 3′ -ends were performed. Two reaction mixtures were there-

fore set up, each containing 4 μl of 'Big Dye' reaction mix, 3.0 μl of DNA, and 0.5 μl of one of the diluted primers (~ 3.2 pmol). The time programme used for the cycle sequencing consisted of 25 cycles of 96℃, 105 s→ 50℃, 55 s→ 60℃, 4 min. Ethanol precipitation was used to purify the extension products after cycle sequencing: after transferring all of the extension product into microcentrifuge tubes, 10 μl deionised H_2O, 40 μl 95% ethanol, and 2 μl of 3M sodium acetate were added and mixed. These were left at room temperature for 15 min before centrifugation (20 min, at 20,000 × *g*). The DNA pellet was aspirated and sequencing was performed in an Automated DNA Analysis system, at the University New South Wales, Australia.

Polysaccharides

Dried tissue (100 mg) of fruit body or the mycelium of *Ganoderma* were refluxed and extracted by immersion with 25ml 80% ethanol in hot water for 4 h. The precipitates were resuspend with 25 ml water and immersed in hot water for 2 h. The supernatant fractions were combined and assay for total polysaccharides (Anthrone method).

Monosaccharide

Dried tissue (100 mg) were extracted in 5 ml cold solvent (methanol : chloroform : water = 60 : 25 : 15) with a pestle and mortar. The mixture was vortexed and centrifuged at 3500 ~ 4000 rpm for 15 min. After volume determination of the supernatant was stored in a vial at 4℃ until analysis. Sugars were analysed as their TMSi derivatives using gas chromatography (GC) by the methods of Ford (1979). Inositol was used as an internal reference, as it is well separated from other sugars. A mixture of 0.40 ml of the extract supernatant and 0.1 ml inositol solution (1 mg/ml, in 20% methanol), was dried in a 1 ml vial, and 0.1 ml of the derivatizing reagent [a mixture of trimethylsylil imidazole and pyridine (1:2, *V/V*)] was added. The vial was vortexed rapidly for a few seconds to dissolve the sugars and left at room temperature overnight to complete the reaction. Samples (1 μl) were analysed by GC (Gas Chromatography, Shimadzu Model GC-17A) and a SPB™-1701 capillary column (30 m × 0.25 mm ID) was used for sugars analysis. The GC was calibrated every day with standard solutions containing 50 μg each of galactose, mannose, rhamnose, xylose, arabinose, fucose, glucose and *myo-inositol*. Temperature of column is 130 ~ 250 ℃, injector 225 ℃ and detector 300 ℃. Carrier gas was hydrogen at a flow rate of 30 ± 1 ml·min^{-1}, split 1:17.

Soluble protein

Tissue extract was prepared modifying a method from Hawkins and Boudet (1994). Dried tissue was ground in 1 ml extraction buffer (100 mM Na Ascorborte, 250 mM Bicine pH 8.0, 1 mM EDTA, 5% ethyl glucose, 10 mM $MgCl_2$ and 20 mM $NaHCO_3$). The extract was centrifuged (20 000 *g* for 10 min) and the pellet resuspended with 1 ml extraction buffer and recentrifuged. The supernatant fractions were combined and assayed for protein (Bradford 1976).

Fatty acid profile

The standard procedure used for analysing the fatty acid contents of plants was as following. Fatty acids were extracted by a method modified from Lepage and Roy (1986) and described in details by Liu et al. (2000). Dried tissue was ground in a mortar and pestle with 6 ml of chloroform/methanol (2:1, *V/V*) containing internal standard (IS, C21:0, Sigma USA, 0.2 mg/ml) and 5% butylhydroxytoluene (BHT) to prevent oxidation of fatty acids. The mixture was vortexed and centrifuged at 3,000 *g* for 10 min at 5℃. The lower phase (1ml) was collected and evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 2 ml of methanol/toluene (4:1, *V/V*) and acetyl chloride (0.2 ml) was slowly added while vigorously stirring. The mixture was heated at 100℃ for 1 h, then cooled in water and the reaction stopped by addition of 6% K_2CO_3 solution (5 ml). The samples were vortexed, centrifuged, and the upper phase was collected prior to GC analysis.

Samples (1μl) were injected via an autosampler onto a fused-silica capillary column (J & W Scientific; DB-Wax; 30 m × 0.25 mm ID; 25 μm film thickness) in a Shimadzu Model GC-17A GC fitted with a FID detector and eluted with H_2 at 30 ± 1 ml/min with a split ratio of 1:17. The injector and de-

tector were heated to 250℃. The column was temperature programmed from 150℃ (hold 1 min) to 180℃ at 25℃/min, then to 220℃ (hold 3 min) at 2.5℃/min and then to 250℃ (hold 4 min) at 15℃/min. Fatty acid methyl esters were identified by comparing GC retention times with those of a mixture of standard fatty acids (Sigma, USA). Fatty acids were quantified using peak areas, against internal standard.

Statistics

All the experiments were repeated at 3 ~ 5 times and data were analysed by general ANOVA and t-Test (Statistica Version 4.0; StatSoft, Inc. USA). Mean values are given in the table and figure.

RESULTS AND DISCUSSION

ITS rDNA analysis to *Ganoderma* taxonomy

Ribosomal DNA (rDNA) sequence has been widely used to discriminate fungal taxonomy at the family, generic and sub-generic level (Ueng et al. 1998). The aligned ITS-1 and ITS-2 rDNA sequences of *G. nt* comparing with others are shown in Figure 3.

G. lucidum GGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCATGTGCACG **55**

QFRI8647.1*G.*sp ---------+++ **46**

DAR73780 *G.*sp ---------+++**46**

G. nt +A+++**55**

G. lucidum CCCTGCTCATCC ACTCTACACCTGTGCACTTACTGTGGGCTTCAGATCGTAAAAC
110

QFRI8647.1*G.*sp ++++++++++G+++++++++++++++++++++++++++++++++++++++ **101**

DAR73780 *G.*sp ++++++++++G+++++++++++++++++++++++++++++++++++++++ **101**

G. nt ++++++++++G+++++++++++++++++++++++++++++++++++++++ **110**

G. lucidum GGGTCCCTTTACCGGGCTTGCGGAGCGTGTCTGTGCCTGCGTTTATCACAAACTC
165

On the base of ITS sequence, *G. nt* is exactly same as the species DAR73780 *G.* sp collected in Indonesia, where is close to Darwin in geographic positions. Only one base pair in both ITS-1 and ITS-2 was different between *G. nt* and QFRI8647.1 *G.* sp which collected in Queensland, Australia. Comparing between *G. nt* and *G. lucidum* varied at 11 sites (base pair) in total. This was confirmed further that *G . nt* is a new *Ganoderma* species to be named rather *G. lucidum* (Smith and Sivasithamparam, 2000).

Polysaccharides

Polysaccharide contents in fruit bodies and mycelia of *G. nt* and *G. lucidum* were compared (Figure 4). Fruit bodies had lower concentration of polysaccharides (10 ~ 12.9 mg/g dry mass) than cultured mycelia. Liquid shaking cultured mycelia had the highest polysaccharides content (138 mg/g).

Monosaccharides

Monosaccharide contents in *G. nt* and *G. lucidum* showed in Table 1. Glucose, mannose and rhamnose are the main sugars in these samples. Same as polysaccharide content in samples, fruit bodies had lower total sugar contents (~ 13.5 mg/g dry mass) than cultured mycelia, and liquid shaking cultured mycelia had highest polysaccharides content (128 mg/g).

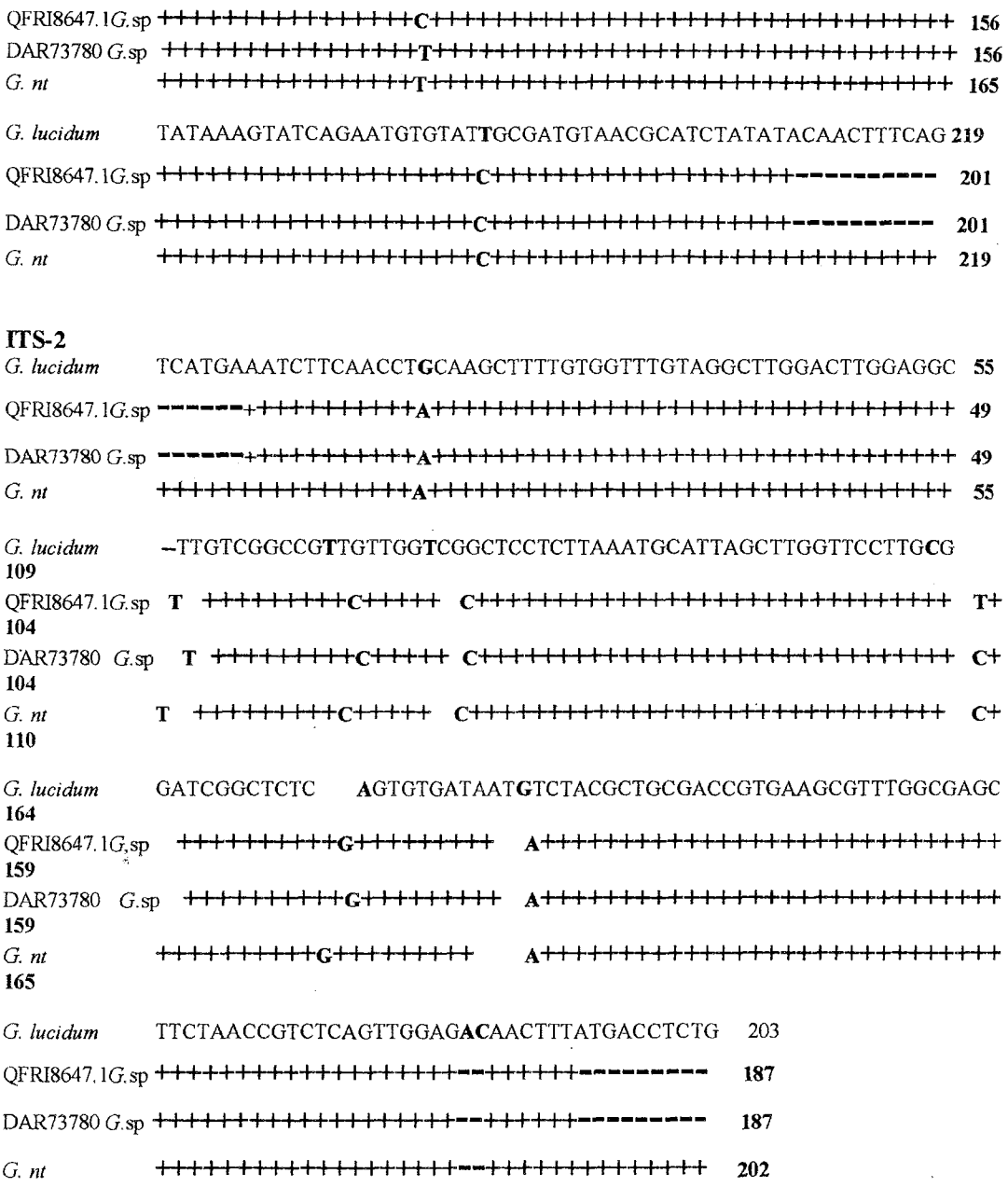

Figure 3. Comparison of aligned sequences of the ITS-1 and ITS-2 regions of rDNA in *Ganoderma* genus. Alignment gaps are indicated by dashes and conserved bases by plus sign. The DNA sequence from left to right reads from 5′- to 3′-. The sequence of ITS-1 and ITS-2 in *G. lucidum* obtained from EMBL (accession no: ITS-1/ITS-2 X78743/X7876), QFRI8647.1 *G.* sp (collected in Queensland, Australia) and DAR73780 *G.* sp (collected in Stump, Indonesia) from Smith and Sivasithamparam (2000).

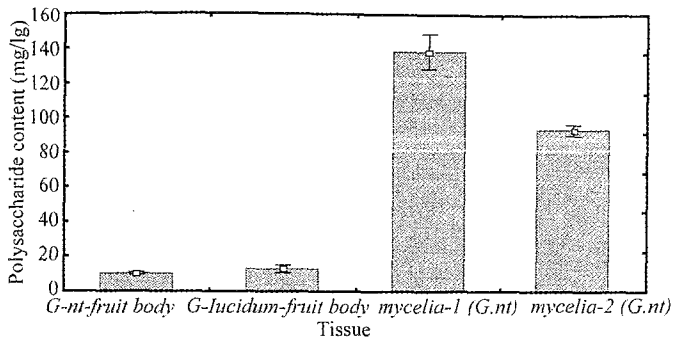

Figure 4. Polysaccharide concentrations (mg/g dry mass) in fruit bodies and cultured mycelia of *Ganoderma*. Mycelia 1- shaking culture; Mycelia 2- static culture

Table 1. Comparison of monosaccharide contents (mg/g dry mass) in fruit bodies and cultured mycelia of *Ganoderma*

Monosaccharide	Fruit body *G. nt*	Fruit body *G. lucidum*	Mycelia 1 *G. nt* (shaking culture)	Mycelia 2 *G. nt* (static culture)
Arabinose	0.577 ± 0.317	0.474 ± 0.292	0.422 ± 0.267	0.382 ± 0.234
Galactose	0.262 ± 0.039	0.107 ± 0.121	3.054 ± 0.296	1.082 ± 0.459
Glucose	6.367 ± 0.454	1.527 ± 0.342	128.1 ± 2.265	64.07 ± 4.163
Fucose	0.179 ± 0.014	0.053 ± 0.022	0.039 ± 0.001	0.377 ± 0.221
Mannose	3.423 ± 0.358	11.32 ± 0.491	10.04 ± 0.955	29.02 ± 3.407
Rhamnose	2.467 ± 0.090	0.253 ± 0.131	5.755 ± 0.150	5.930 ± 0.639
Xylose	0.208 ± 0.061	n.d.	0.974 ± 0.106	0.711 ± 0.101
Total sugars	13.49 ± 0.217	13.74 ± 0.111	148.3 ± 3.146	101.6 ± 1.786

Soluble protein

Soluble protein contents in *G. nt* and *G. lucidum* showed in Figure 5 and ranged 8.6 ~ 23.1 mg/g dry mass. Liquid static cultured mycelia had highest soluble protein content.

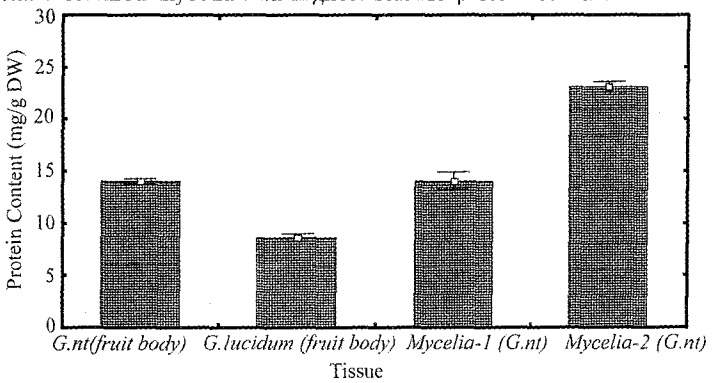

Figure 5. Soluble protein content (mg/g dry mass) in fruit bodies and cultured mycelia of *Ganoderma*. Mycelia 1- shaking culture; Mycelia 2- static culture

Fatty acids

Total fatty acid contents in *G. nt* and *G. lucidum* showed in Table 2 and ranged 4.35 ~ 6.14 mg/g dry mass. 10:0, 16:0 and18:1n9 are main contents in fruit bodies of *Ganoderma* and 10:0, 16:0 and

18:2n6 are main contents in cultured mycelia. Liquid shaking cultured mycelia had a trace of 18:3n3, α-linolenic acid.

Table 2. Comparison of fatty acid contents (mg/g dry mass) in fruit bodies and cultured mycelia of *Ganoderma nt* and *Ganoderma lucidum*

(mg/g)	Fruit body *G. nt*	Fruit body *G. lucidum*	Mycelia-1 *G. nt*	Mycelia-2 *G. nt*
10:0	1.446 ± 0.023	1.758 ± 0.069	2.109 ± 0.076	2.220 ± 0.009
12:0	0.006 ± 0.001	0.006 ± 0.001	0.007 ± 0.002	0.008 ± 0.002
14:0	0.022 ± 0.002	0.011 ± 0.001	0.049 ± 0.002	0.023 ± 0.001
15:0	0.041 ± 0.002	0.151 ± 0.149	0.084 ± 0.001	0.034 ± 0.004
16:0	1.164 ± 0.039	0.522 ± 0.044	1.076 ± 0.049	0.476 ± 0.001
16:1	0.025 ± 0.002	0.036 ± 0.007	0.014 ± 0.003	0.021 ± 0.007
18:0	0.208 ± 0.015	0.067 ± 0.009	0.152 ± 0.003	0.078 ± 0.005
18:1c	1.906 ± 0.033	0.999 ± 0.108	0.253 ± 0.010	0.334 ± 0.081
18:1t	0.033 ± 0.001	0.068 ± 0.004	n.d.	0.053 ± 0.005
18:2	0.669 ± 0.026	0.667 ± 0.079	2.247 ± 0.234	1.155 ± 0.194
18:3	n.d.	n.d.	0.020 ± 0.001	n.d.
22:0	0.039 ± 0.002	0.027 ± 0.009	0.068 ± 0.004	0.064 ± 0.005
20:2	0.006 ± 0.001	0.028 ± 0.002	n.d.	n.d.
24:0	0.050 ± 0.006	0.014 ± 0.001	0.055 ± 0.002	0.017 ± 0.004
Total FA	5.615 ± 0.033	4.354 ± 0.283	6.135 ± 0.228	4.589 ± 0.091

Mycelia 1- shaking culture; Mycelia 2- static culture

CONCLUSION

An Australian species of *Ganoderma* genus (temporally named *G. nt*) mistaken for *G. lucidum*, a well-known herbal medicine, was examined with internal transcribed spacer ribosomal DNA (ITS rDNA) sequence as an aid to the taxonomy. Variation between *G. nt* and *G. lucidum* in the ITS rDNA sequence was 2% ~ 4%. Compared with *G. lucidum*, *G. nt* had similar contents in polysaccharides and monosaccharides on dry mass base in fruit body. However, *G. nt* fruit body had higher soluble protein and fatty acids contents than *G. lucidum*. Mycelia of *G. nt* obtained from shaking and static cultures had higher contents of polysaccharides and monosaccharides than fruit bodies. As this unnamed Australian species of *Ganoderma* - *G. nt* had similar or higher nutrient value compared to *G. lucidum*, it is necessary to exam the bio-activity and stimulating effect on the immune system in animals of both fruit body and mycelium. Since *G. lucidum* is not native to Australia and live fungus cannot be imported, identification and evaluation of Australian species of *Ganoderma* resembling *G. lucidum* presents a significant opportunity to develop it as a commercial source of bio-active nutrients for the production of dietary supplements.

ACKNOWLEDGMENT

This work was supported by an Australian Research Council grant to the Smart Foods Centre.

REFERENCES

Blackford FW. 1944. A *Ganoderma* root rot of citrus. *Queensland Journal of Agricultural Science*, 1: 77 ~ 81

Brower V. 1988. Nutriceuticals: Poised for a healthy slice of the healthcare market? *Nature Biotechnology*, 16:728 ~ 731

Chang ST and Buswell JA. 1996. Mushroom nutriceuticals. *World J Microb Biotechnol*, 12:473 ~ 476

Chang ST and Buswell JA. 1999. *Ganoderma lucidum* (Curt.:Fr.) P. Karst. (Aphyllophoromycetideae) -A mushrooming

medicinal mushroom. *International J Medicinal Mushrooms*, 1:139 ~ 146
Cooke MC. 1883. Fungi Australian: Melbourne, Australia.
Lepage G and Roy GC. 1986. Direct transesterification of all classes of lipid in a one step reaction. *J Lip Res* 27:114 ~ 120
Lin ZB 1996. Physiological effects of *Ganoderma lucidum*. In: Lin ZB ed. Modern research on *Ganoderma lucidum* (in Chinese). Beijing: Beijing Medical University Press, 148 ~ 177
Liu LX, Howe P, Zhou YF, Xu ZQ, Horcok C and Zhang R. 2000. Fatty Acids and β-Carotene in Australian Purslane (*Portulaca oleracea*) Varieties. *J Chromatography* (A). 893: 207 ~ 213
Mizuno T. 1995. *Food Reviews International*, 7 ~ 21, 23 ~ 61, 151 ~ 166
Smith BJ and Sivasithamparam K. 2000. Internal transcribed spacer ribosomal DNA sequence of five species of *Ganoderma* from Australia. *Mycological Research*, 104:943 ~ 951
Wasser PS, Nevo E, Sokolov D, Reshetnikov S and Timor-Tismenetsky M. 2000. Dietary supplements from medicinal mushrooms: Diversity of types and variety of regulations. *Intl J Medicinal Mushrooms*, 2:32 ~ 37
Yeh ZY and Chen ZC. 1990. Preliminary investigations of *Ganoderma australe* in Taiwan. *Taiwania*, 35: 127 ~ 141
Zeisel SH. 1999. Regulation of "Nutriceuticals". *Science*, 285: 1853 ~ 1855