Journal of Mushrooms 한국버섯학회지 Research Article

Microscopical observation and randomly amplified polymorphic DNA (RAPD) analysis of artificially cultivated *Ganoderma applanatum*

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ABSTRACT: This study investigated the microscopic characteristics and genetic relationships of *Ganoderma applanatum* fruiting bodies. Basidiospores were brown, ellipsoid, and had one or two large vacuoles and a double wall. The surface of basidiospores was smooth or wrinkled and most had numerous small and shallow holes. The length and width of basidiospores of *Ganoderma applanatum* isolates GBGA-01, GBGA-02, ASI 50167, ASI 52821, ASI 52822, ASI 52823, and ASI 53399 were on average 7.6×4.8 μm, 7.9×4.6 μm, 7.7×4.9 μm, 8.2×5.3 μm, 7.7×5.0 μm, 8.0×4.9 μm, and 7.9×4.9 μm, respectively. In contrast, the basidiospores of *Ganoderma lucidum* isolate ASI 7125 were 7.7×5.2 μm. Using the universal ITS1/ITS4 primer set, the ITS region of the isolates were amplified and sequenced. The ITS sequences were very closely related to *G. applantum* isolate GBGA-01, GBGA-02, ASI 50167, ASI 52821, ASI 52822, ASI 52823 and ASI 53399, but were not the same species. Whereas, *G. lucidum* isolate ASI 7125 belongs to different group.

KEYWORDS: Basidiospore, *Ganoderma applanatum*, ITS sequences

INTRODUCTION

A stalked mushroom with porous hymenium, *Ganoderma lucidum* (Fr.) Karst, causes white rot of wood, decomposing lignin as well as cellulose and related polysaccharides, as the fungus decays hard wood such as oak, maple sycamore and ash (Hepting, 1971; Blanchette, 1984). It is estimated that there are 140,000 species of mushrooms worldwide, yet only 10 % have been identified (Kirk *et al*., 2001). Mushrooms have long been valued as edible and medicinal resources. *Ganoderma applanatum* (Pers.) Karst, which belongs to Polyporaceae of basidiomycetes, grows spontaneously on the branches of the broad leaf tree. Specifically, *G. applanatum* forms semicircular carpophores on the branch in parallel. *G. applanatum* is found worldwide, including in Korea (Park, 1991), where it has long been used as a medicine for the treatment of various human tumorigenic diseases (Kim *et al*., 1980). The mushroom reportedly contains a variety of biologically active components, including bitter triterpenoids (Nishitoba *et al*., 1988, 1989), alnusenone, friedelin (Protiva *et al*., 1980), α-D-glucan and β-D-glucan (Mizuno *et al*., 1981, 1985; Usui *et al*., 1983). Study for culture conditions for mycelial growth and artificial cultivation of *G. applanatum* was conducted (Jo *et al*., 2009, 2023). In addition, efficacy tests related to antidiabetic using specific ingredients of the *G. applanatum* mushroom extract have been reported (Shim *et al*., 2004; Jung *et al*., 2005; Lee *et al*., 2006). This study was conducted to investigate the differences between artificially cultivated *G. applanatum* fruiting bodies and use it as basic data for cultivating new varieties.

J. Mushrooms 2024 June, 22(2):48-52
http://dx.doi.org/10.14480/JM.2024.22.2.48
Print ISSN 1738-0294, Online ISSN 2288-8853
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Received June 18, 2024
Revised June 20, 2024
Accepted June 24, 2024

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MATERIALS AND METHODS

1. Microscopical observation

The formation of AFSs and non-basidiocarpous basidiospores in *Ganoderma* spp. was monitored under a stereo microscope and light microscope (Eclipse 80i). Cultures were also observed under a scanning electron microscope (SEM, LEO 1450VP). For SEM observation, about 5 mm

Table 1. List of *Ganoderma* spp. strains used in this study.

Scientific name	Strain name	Korean common name	Origin culture	Origin
Ganoderma applanatum	Samjung	잔나비불로초	GBGA-01	Gyeongbuk Agricultural Technology Administration, Korea
Ganoderma applanatum	-	잔나비불로초	GBGA-02	Gyeongbuk Agricultural Technology Administration, Korea
Ganoderma applanatum	-	잔나비불로초	ASI 50167	Rural Development Administration, Korea
Ganoderma applanatum	-	잔나비불로초	ASI 52821	Rural Development Administration, Korea
Ganoderma applanatum	-	잔나비불로초	ASI 52822	Rural Development Administration, Korea
Ganoderma applanatum	-	잔나비불로초	ASI 52823	Rural Development Administration, Korea
Ganoderma applanatum	-	잔나비불로초	ASI 53399	Rural Development Administration, Korea
Ganoderma lucidum	-	영지버섯	ASI 7125	Rural Development Administration, Korea

square plugs of AFSs were removed from cultures with a razor blade. Samples were fixed using 2.5 % glutaraldehyde and post-fixed in 2 % osmium tetroxide (Mims and Seabury, 1989; Adaskaveg and Gilbertson, 1986). These sample were then dehydrated in a series of 30 to 100 % ethanol, and with a series of the ethanol 50, 75, and 100 % amylacetate. Finally, the plugs were dried in carbon dioxide using a critical-point dryer (SC7620, POLARON), mounted on specimen stubs with adhesive silver pasts, and coated with gold to a thickness of about 15 nm using a sputter multicoater (R-266, POLARON).

The isolates of *G. applanatum* used in this study are listed in Table 1. *G. applanatum* ASI 50167, ASI 52821, ASI 52822, ASI 52823, ASI 53399 and *G. lucidum* ASI 7125 were obtained from the Rural Development Administration in Korea. *G. applanatum* GBGA-01 and GBGA-02 were collected in the wild. All isolates were maintained on Potato Dextrose Agar (PDA).

2. Randomly amplified polymorphic DNA (RAPD) analysis

Preparation of fungal DNA

Total genomic DNA was prepared by a modification of the mini-preparation method described by Yoder (1988). Mycelium was obtained by growing *Ganoderma* spp. in CM broth at 25 ℃ for 5 days, followed by harvesting, washing with H_2O, and freeze-drying.

Approximately 30 mg of freeze-dried mycelium was ground to fine powder under liquid N_2 in a 1.5 ml Eppendorf tube, and 400 or 100 μl extraction buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris-Cl, pH 7.4 and 0.5 % SDS) was added. This suspension was mixed well and extracted twice with phenol/$CHCl_3$, and once with $CHCl_3$. One tenth volume of CH_3COONa (3 M, pH 5.2) was added to the aqueous phase and nucleic acid was precipitated with ethanol (2 volumes, -20 ℃). After centrifugation at 12,000 rpm for 10 min, the precipitate was washed once with 70 % ethanol (500 μl, room temperature), dried under vacuum and dissolved in 100 μl of TE buffer. RNA was removed by adding RNase at a final concentration of 200 ng/μl and incubating for 60 min, at 37 ℃ before second phenol/$CHCl_3$ extraction. The concentration of DNA was determined by running a sample of the DNA on a 0.7 % agarose gel, staining with ethidium bromide, and comparing the intensity of the DNA band to the intensities of a set of lambda DNA standards (adjusted to concentrations of 20, 50, 100 and 200 ng/well) under UV light (254 nm).

Amplification conditions

The Polymerase chain reaction (PCR) amplification conditions used are based on those of Williams *et al*. (1990) and Welsh and McClelland (1990) using modification as described by Crowhurst *et al*. (1991). Amplification reactions were carried out in a final volume of 20 μl containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-Cl (pH 8.3), 125 mM of each dNTP, 200 nM primer, 1 units of Takara Taq DNA polymerase (Takara) and 25 to 50 ng of genomic DNA. PCRs were performed on a Perkins-Elmer/Cetus Gene Amp PCR system 9600 (Norwalk, CT). The temperature cycle used for amplification was 3 min at 94 ℃, followed by 40 cycles of 1 min at 94 ℃, 1 min at 35 ℃ and 3 min at 72 ℃. Primers used were GT-02 (5'-TGGTGGGTCC-3'), and RC-06 (5'-ACATAGACGG-3'). Both primers were purchased from Nippon Gene Co. Ltd.

RAPD analysis

RAPDs generated by single primer PCR were used to

Table 2. Basidiopore size of *Ganoderma* spp. strains generated from basidiocarps on oak sawdust bottle.

Scientific name	Origin culture	Length (mm)	Width (mm)	Range (mm)	SSI*
Ganoderma applanatum	GBGA-01	7.6±0.6	4.8±0.2	6.8~8.8×4.5~5.2	63
Ganoderma applanatum	GBGA-02	7.9±0.5	4.6±0.4	7.0~8.4×4.2~5.5	58
Ganoderma applanatum	ASI 50167	7.7±0.3	4.9±0.2	7.2~8.2×4.4~5.2	64
Ganoderma applanatum	ASI 52821	8.2±0.6	5.3±0.5	7.4~9.2×4.6~6.2	65
Ganoderma applanatum	ASI 52822	7.7±0.5	5.0±0.3	7.0~8.5×4.5~5.4	65
Ganoderma applanatum	ASI 52823	8.0±0.6	4.9±0.4	7.2~9.3×4.5~5.8	61
Ganoderma applanatum	ASI 53399	7.9±0.5	4.9±0.2	7.2~8.7×4.6~5.1	62
Ganoderma lucidum	ASI 7125	7.7±0.5	5.2±0.4	7.0~8.7×4.8~5.8	68

*SSI (spore shape index) = diam × 100 / length

compare relatedness of isolates. Amplified products were run on a 1 % agarose gel and the molecular size of each fragment was estimated using a standard curve of migration versus log molecular size of *Hind*III digested bacteriophage lambda and pGEM. For each isolate, a data record was constructed in which each band of a particular molecular weight, as generated by each primer, was represented as either being present "1" or absent "0". The portion of common fragments between two isolates was calculated using Dice's coefficient:

$$D=2n_{xy}/(n_x+n_y)$$

where n_{xy} is the number of common fragments, and n_x and n_y are the number of fragments amplified from each isolate (Sneath and Sokal, 1973).

Dice's similarity matrixes were analyzed by an UPGMA (unweighted paired group method using arithmetic means) clustering performed with the program NTSYS-pc (numerical taxonomy system using multivariate statistical programs) (Rohlf, 1988).

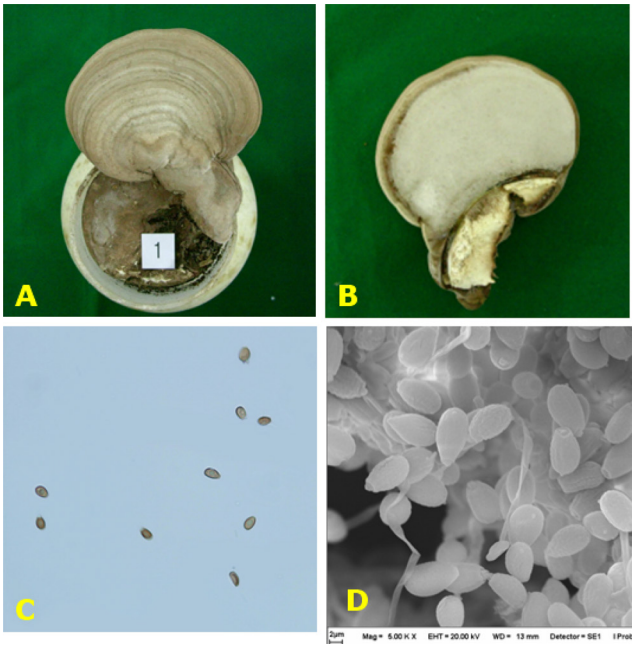

Fig. 1. Morphological characteristics of *G. applanatum* GBGA-01 fruitingbody. A: Basidiocap, B: Hymenial pores, C: Basidiospores observed with light microscope (×400), D: Basidiospores observed with SEM (×5,000).

RESULTS AND DISCUSSION

Microscopical observation

Basidiospores were brown, ellipsoid, and had one or two large vacuoles and a double wall. The surface of basidiospores was smooth or wrinkled and most had numerous small and shallow holes. The surface of immature basidiospores on basidia was wrinkled only. Basidiospores were truncated to narrowly rounded at the apex with an eccentric hilar appendix on a rounded spore base. Length and width of basidiospores of *Ganoderma applanatum* isolate GBGA-01, GBGA-02, ASI 50167, ASI 52821, ASI 52822, ASI 52823 and ASI 53399 were 7.6×4.8 μm, 7.9×4.6 μm, 7.7×4.9 μm, 8.2×5.3 μm, 7.7×5.0 μm, 8.0×4.9 μm and 7.9×4.9 μm on average. The above was similar to the following report. Microscopically, the cultivated Chinese '*G. lucidum*' (Ling-zhi) was characterised by hymeniodermiformic cutis, Bovista-type ligative hyphae and strongly echinulate basidiospores of 6.5–8.5×5.0–6.5 μm sine myxosp (Wang *et al*., 2012). *Ganoderma lucidum* isolate ASI 7125 was 7.7×5.2 μm. The mean spore shape index (ratio of spore length to width) of *Ganoderma applanatum* isolate GBGA-01 and *Ganoderma lucidum* isolate ASI 7125 were 63 and 68, respectively. The other morphological characteristics such as surfaces of structures, color and shape were almost the same (Table 2, Figs. 1, 2, 3).

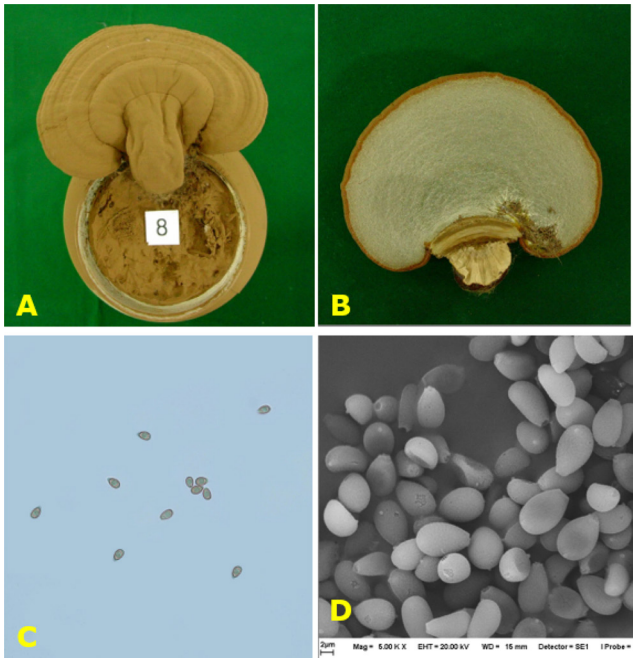

Fig. 2. Morphological characteristics of *G. lucidum* ASI 7125 fruitingbody. A: Basidiocap, B: Hymenial pores, C: Basidiospores observed with light microscope (×400), D: Basidiospores observed with SEM (×5,000).

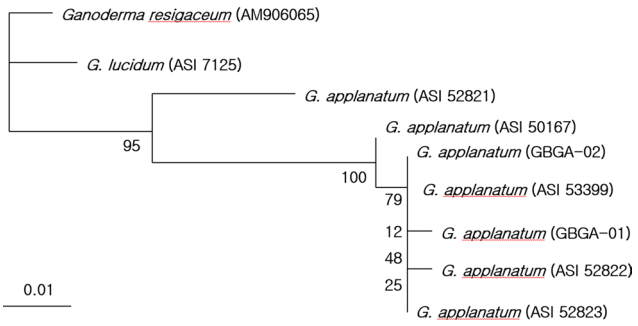

Fig. 4. Neighbor-joining phylogenetic tree of *Ganoderma* spp. strains based on concatenated sequences of internal transcribed spacer (ITS).

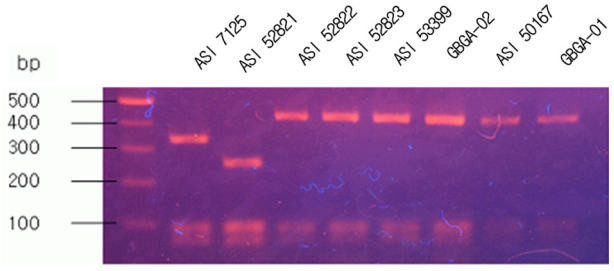

Fig. 5. Agarose gel electrophoresis of PCR-amplified rDNA-ITS regions of *Ganoderma* spp. strains.

Randomly amplified polymorphic DNA (RAPD) analysis

Using the universal ITS1/ITS4 primer set, the ITS region of the isolates were amplified and sequenced. The ITS sequences were very closely related to *G. applantum* isolate GBGA-01, GBGA-02, ASI 50167, ASI 52821, ASI 52822, ASI 52823 and ASI 53399, but were not the same species. Whereas, *G. lucidum* isolate ASI 7125 belongs to different group (Figs. 4, 5).

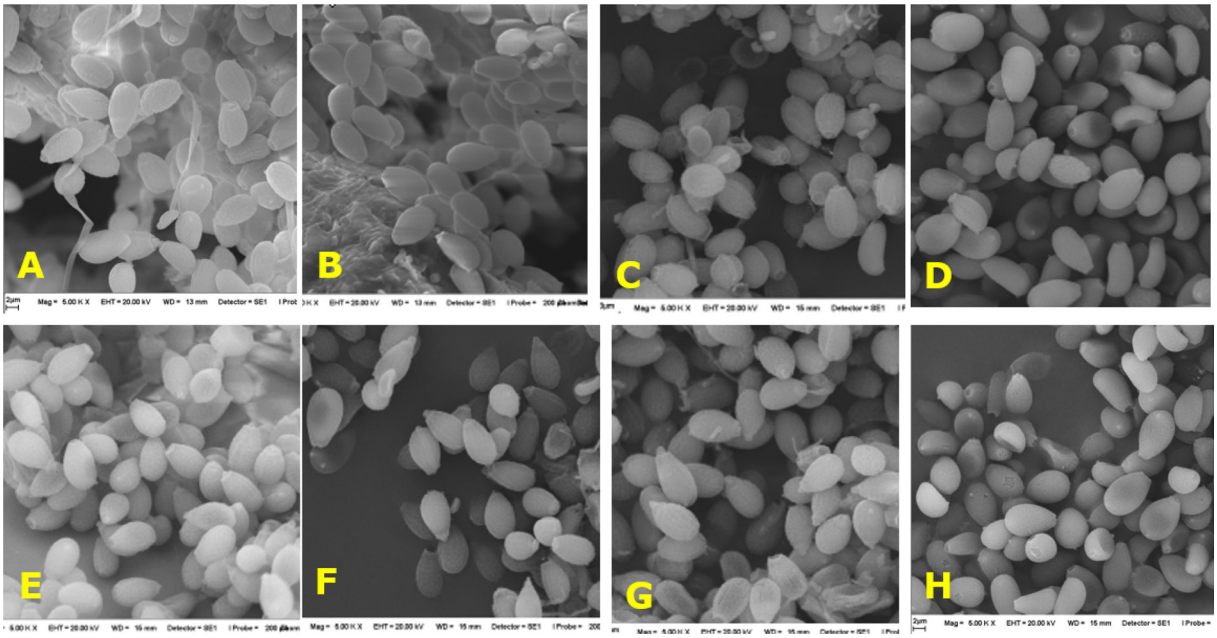

Fig. 3. Basidiospores observed with SEM (×5,000).
A: GBGA-01, B: GBGA-02, C: ASI 50167, D: ASI 52821, E: ASI 52822, F: ASI 52823, G: ASI 53399, H: ASI 7125.

CONCLUSION

Length and width of basidiospores of *Ganoderma applanatum* isolate GBGA-01, GBGA-02, ASI 50167, ASI 52821, ASI 52822, ASI 52823 and ASI 53399 were 7.6×4.8 μm, 7.9×4.6 μm, 7.7×4.9 μm, 8.2×5.3 μm, 7.7×5.0 μm, 8.0×4.9 μm and 7.9×4.9 μm on average. *Ganoderma lucidum* isolate ASI 7125 was 7.7×5.2 μm. The mean spore shape index (ratio of spore length to width) of *Ganoderma applanatum* isolate GBGA-01 and *Ganoderma lucidum* isolate ASI 7125 were 63 and 68, respectively. The other morphological characteristics such as surfaces of structures, color and shape were almost the same. Using the universal ITS1/ITS4 primer set, the ITS region of the isolates were amplified and sequenced. The ITS sequences were very closely related to *G. applantum* isolate GBGA-01, GBGA-02, ASI 50167, ASI 52821, ASI 52822, ASI 52823 and ASI 53399, but were not the same species. Whereas, *G. lucidum* isolate ASI 7125 belongs to different group. We hope the results of the study will help foster new varieties of *G. applantum*, a medicinal mushroom in the future.

ACKNOWLEDGEMENTS

This study was conducted with the support of National Joint Agricultural Research Project of the RDA (Project No. 20080301-030-034-001-05-00), Republic of Korea.

REFERENCES

Adaskaveg JE, Gilbertson RL. 1986. Cultural studies and genetics of sexuality of *Ganoderma lucidum* and *G. tsugae* in relation to the taxonomy of the *G. lucidum* complex. *Mycologia*. 78: 694-705.

Blanchette RA. 1984. Screening wood decayed by white rot fungi for preferential lignin degradation. *Appl Environ Microbiol*. 48: 647-653.

Crowhurst RN, Hawthorne BT, Rikkerink EHA, Tempelton MD. 1991. Differentiation of *Fusarium solani* F. cucurbitae race 1 and 2 by random amplification of polymorphic DNA. *Curr. Genet*. 20: 391-396.

Hepting GH. 1971. Disease of forest and shade trees of the United States. U. *S. Dept. Agric., Agric. Handb.* 386: 1-658.

Jo WS, Bae SH, Yoo YB. 2023. Characteristics of artficially cultivated *Ganoderma applanatum* fruitingbody. *J Mushrooms.* 21: 47-52.

Jo WS, Cho YJ, Cho DH, Park SD, Yoo YB, Seok SJ. 2009. Culture Conditions for the Mycelial Growth of *Ganoderma applanatum. Mycobiology*. 37: 94-102.

Jung SH, Lee YS, Shim SH, Lee S. Shin KH, Kim JS, Kim Ys, Kang SS. 2005. Inhibitory effects of *Ganoderma applanatum* on rat lens aldose reductase and sorbitol accumulation in streptozotocin-induced diabetic rat tissue. *Phytother Res*. 19: 477-480.

Kim BK, Chung HS, Chung KS, Yang MS. 1980. Studies on the antineoplastic components of Korean basidiomycetes. *Kor. J. Mycol.* 8: 107-113.

Kirk PM, Cannon PF, David JC, Stalpers JA. 2001. Ainsworth and Bisby's dictionary of the fungi. *9th edn., CAB International, Wallingford.*

Lee SY, Shim SH, Kim JS, Kang SS. 2006. Constituents from the fruiting bodies of *Ganoderma applanatum* and their aldose reductase inhibitory activity. *Arch Pharm Res*. 29: 479-483.

Mims CW, Seabury F. 1989. Ultrastructure of Tube Formation and Basidiospore Development in *Ganoderma lucidum*. *Mycologia*. 81(5): 754-764.

Mizuno T, Hayashi K, Arakawa M, Shinkai K, Shimizu M, Tanaka M. 1981. Host-mediated antitumor polysacchrides. III. Fractionation, chemical structure, and anti-tumor activity of water-soluble homoglucans isolated from kofukisarunokoshikake, the fruit body of *Ganoderma applanatum*. *Shizuoka Daigaku Nogakubu Kenkyu Hokoku* 31: 49-64.

Mizuno T, Suzuki E, Maki K, Tamaki H. 1985. Fractionation, cemical modification and antitumor activity of water-insoluble polysaccharides of the fruiting body of *Ganoderma lucidum*. *Nippon Nôgeikagaku Kaishi.* 59: 1143-1151.

Nishitoba T, Goto S, Sato H, Sakamura S. 1989. Bitter triterpenoids from the fungus *Ganorderma applanatum*. *Phytochemistry*. 28: 193-197.

Nishitoba T, Sato H, Oda K, Sakamura S. 1988. Novel triterpenoids and a sterol from the fungus *Ganoderma lucidum*. *Agric. Biol. Chem.* 52: 211-216.

Park WH. 1991. Colored fungi of Korea. *Kyo-Hak Publishing Co., Ltd.* 504pp.

Protiva J, Skorkovska H, Urban J, Vystrcil A. 1980. Triterpenes LXIII. Triterpenes and steroids from *Ganoderma applanatum*. *Coll Czech Chem. Commun.* 45: 2710-2713.

Rohlf, F. J. 1988. NTSYS-pc: Numerical taxonomy and multivariate analysis system. Version 1.80. Exeter Software, Setauket, New York.

Shim SH, Ryu J, Kim JS, Kang SS, Xu Y, Jung SH, Lee YS, Lee S, Shin KH. 2004. New lanostane-type triterpenoids from *Ganoderma applanatum*. *J Nat Prod*. 67: 1110-1103.

Sneath PHA, Sokal RR. 1973. Numerical taxonomy: The principles and practice of Numerical classification. *Freeman, Sanfrancisco*. 573.

Usui T, Iwasaki Y, Mizuno T, Tanaka M, Shinkai K, Arakawa M. 1983. Isolation and characterization of antitumor active β-D-glucans from the fruit bodies of *Ganoderma applanatum*. *Carbohydr. Res.* 115: 273-280.

Wang XC, Xi RJ, Li Y, Wang DM, Yao YJ. 2012. The Species Identity of the Widely Cultivated Ganoderma,'*G. lucidum*'(Ling-zhi), in China. *PLoS ONE* 7(7): e40857. doi:10.1371/journal.pone.0040857.

Welsh J, Mcclelland M. 1990. Fingerfrinting genomes using PCR with arbitrary primers. *Nucleic Acids res*. 18: 7213-7218.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primes are useful as genetic markers. *Nucleic Acids Res*. 18: 6531-6535.

Yoder OC. 1988. *Cochliobolus heterostrophus*, cause of southern corn leaf blight. In: Ingram DS, Williams PH, eds. Advances in plant pathology. Vol. 6. London, England: *Academic Press*. 93-112.