

Phylogenetic and Biochemical Analysis
of the Reishi Mushroom (*Ganoderma
lucidum*) Populations from AltaiSlynko NM, Blinov AG, Babenko VN, Mihailova SV, Bannikova SV, Shekhovtsov
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CC-BY 4.0**Keywords** Ganodermataceae;
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Abstract

G. lucidum is a typical representative of wood-rotting polypores of the Ganodermataceae family (Basidiomycetes). In Russia, *G. lucidum* is predominantly found in southern regions: in Stavropol and Krasnodar krais, Northern Caucasus, as well as in Altai taiga in logging areas. In this study we investigated the phylogeny of *G. lucidum* specimens from Altai based on the ITS1 ribosomal spacer, and compared them to reishi from other regions of the world. We also studied the phytochemical content of reishi fruit bodies. Results of the screening suggest that ethanol fractions contain mostly flavonoids, phenols, and coumarins; water fractions are dominated by tannins, carbohydrates, and coumarins; and hexane and ethyl acetate extracts, by terpenoids. The main fatty acids were palmitic, oleic, linoleic, and linolenic acids. We found that fruit bodies of Altai *G. lucidum* contained 32.4 mg of phenols per 1 g of extract (in pyrocatechol equivalent), while flavonoids made up 11.1 mg per g (in quercetin equivalent). Polysaccharide content was 10.72% of the absolutely dry substance.

Introduction

Fungi are widely used in traditional medicine. *Ganoderma lucidum* (reishi) has a long history in the pharmacy of China, Korea, Japan, and other countries of Southeast Asia [1]. It is mentioned in the 2000 year old "Holy Book of Miraculous Medicinal Plants" from China. *G. lucidum* contains many biologically active compounds including triterpenes, polysaccharides, peptidoglycans, and ganoderic acids [2,3]. Reishi has much more importance for pharmacy than as a food product; it has a wide range of activities including antitumor, antioxidant, immunomodulating, antiviral, antibacterial, antidiabetic, lipid-lowering, hepatoprotective, gastroprotective, and renoprotective ones [4-8]. Chemical composition of fruit bodies of various *G. lucidum* strains depends on their origin, rearing, and other conditions, and genetic affinity does not correlate with metabolic groupings [9].

G. lucidum is a typical representative of wood-rotting polypores of the Ganodermataceae family (Basidiomycetes). In Russia, *G. lucidum* is predominantly found in southern regions: in Stavropol and Krasnodar krais, Northern Caucasus, as well as in Altai taiga in logging areas. Reishi causes slowly developing white rotting of the trunk. [3].

Scientists recognize many subspecies and varieties of *G. lucidum* based on morphology, and there is currently no clear classification [10-12]. DNA analysis suggest that there is a number of distinct geographical groups [2,3,13]. Classification of *G. lucidum* is exacerbated by the existence of many laboratory strains. Reishi may well be a complex of closely related species, which includes: *Ganoderma tsugae* Murr., *Ganoderma valesiacum* Boud., *Ganoderma oregonense* Murr., *Ganoderma resinaceum* Boud., *Ganoderma pfeifferi* Bres., *Ganoderma oerstedii* (Fr.) Torr., *Ganoderma ahmadii* Stey., *Ganoderma flexipes*, *G. multipileum*, *G. sichuanense*, *G. tropicum* [14].

In this study we investigated the phylogeny of *G. lucidum* specimens from Altai based on the ITS1 ribosomal spacer, and compared them to reishi from other regions of the world. We also studied the phytochemical content of reishi fruit bodies.

Materials and Methods

Studied specimens

Fruit bodies of *Ganoderma lucidum* were collected on logging sites in larch forests in the Northern, Northwestern, and Central physico geographical provinces of the Altai mountain region. Locations in table 1 refer to the nearest settlement.

DNA extraction

A piece of fruit body (1 to 2 g) was ground in liquid nitrogen using a porcelain mortar; 200 µg of the powder was used to extract total DNA using the Dneasy Plant Mini Kit

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(QIAGEN). Fungal-specific primers to the ITS1 region were used: (A) 5'- CCGTAGGTGAACCTGCAGAA -3' and (D) 5'-CGCATTTCGCTGCGTTCTTC -3' [15]. Amplifications were performed in 20 µl volume at the following amplification profile: initial denaturation, 94°C for 3 min, and 30 cycles of 94°C for 30 s, 42°C for 42 s, and 72°C for 1 min [16]. The obtained DNA fragments were visualized in 1% agarose gels made on 1xTAE buffer. Sequencing was performed using BigDye Terminator Ready Reaction Mix (Applied Biosystems) according to manufacturers' instructions

Phylogenetic analysis

Phylogenetic trees were built using the Neighbor Joining algorithm [17]; the validity of the obtained branching pattern was verified using 1000 bootstrap replications [18]. Pairwise Tamura-Nei distances [19.] were calculated using MEGA 3.0 [20].

Determining the composition of biologically active substances

Chemical analysis of Biologically Active Substances (BAS) in fruit bodies of *G. lucidum* included estimation of water and ash content, and obtaining extracts with water and organic solvents (hexane, ethyl acetate, and ethanol). These extracts were used for qualitative and quantitative estimates of various BAS using conventional phytochemical techniques; the results were compared to similar studies.

Water content

To determine water content, chopped reishi were dried at 70°C in an air thermostat until constant weight was achieved.

Ash content

Samples of ~1 g were placed in hot porcelain crucibles and kept in a muffle furnace at 700°C for 2 h, then transferred to a desiccator, cooled and weighed. These procedures were repeated until the difference between two consecutive weighing did not exceed 0.0005 g.

Extraction

Total extraction: Powdered fruit bodies (20.0 g) were extracted in a Soxhlet apparatus for 6 h by ethyl acetate and ethanol, obtaining EA+ and EtOH+ extracts, respectively. Water extraction was performed using 1 l of sodium phosphate buffer (pH 7.0) at 62°C for 16 h in an air thermostat. After cooling, samples were centrifuged at 3000 rpm for 15 min, and the extraction procedure was twice repeated with the sediment. The supernatants were combined, concentrated in a rotor evaporator and adjusted to 200 ml by water, resulting in W+ extract.

Stepwise extraction: Powdered fruit bodies (10.0 g) were extracted by hexane in a Soxhlet apparatus for 6 h, then dried to constant weight in a vacuum desiccator with phosphoric anhydride, and weighed. The same operation was repeated with ethyl acetate, and again with ethanol. Each extract was concentrated in vacuum at 40°C to 100 ml in a Buchi R-210 rotor evaporator (Buchi R-210, Flawil, Switzerland), resulting in G, EA-, and EtOH- extracts. The sediment remaining after ethanol extraction was placed in a 2 l flask containing 1 l of sodium phosphate buffer (pH 7.0), incubated in an air thermostat at 62°C for 16 h, cooled and centrifuged at 3000 rpm for 15 min. This procedure was repeated twice; pooled supernatants were concentrated in a rotor evaporator and adjusted to 100 ml by water, resulting in W- extract.

The remaining insoluble sediment was weighed, and the total mass of extracted material was determined.

Isolation of acid polysaccharides

Acids polysaccharides were isolated as described in Sone et al. [21]. According to this method, 100 ml of 0.1 M NaOH and 20 mg of sodium borohydride were added to 10 g of dried insoluble sediment after water extraction and incubated for 2 h on a magnetic stirrer at room temperature, then centrifuged, and the same procedure was repeated again. The obtained supernatants were pooled, adjusted to pH=7.0 with HCl, concentrated to 50 ml on a Buchi R-210 rotor evaporator (Flawil, Switzerland), and dialyzed against double distilled water for seven days using the Visking (Serva, Germany) dialysis membrane; water was replaced three times at 8 h intervals, then once a day. The dialysate was concentrated to 20 ml using a rotor evaporator; 5 ml aliquotes were used to determine polysaccharide concentration.

Ergosterol extraction

Ergosterol was extracted from the hexane extract as following: 1 ml of methanol was added to 150 mg of the evaporated extract, incubated for 7 days, then filtered. The resulting sediment was dissolved in 5 ml diethyl ether, washed three times with 2 ml of 3% NaOH and three times with 2 ml of sodium bicarbonate, then dried over anhydrous sodium sulfate, filtered, distilled to remove ethers, and recrystallized from methanol.

Phytochemical screening

Phytochemical screening for alkaloids, flavonoids, terpenoids, tannins, saponins, phenols, and coumarin was performed as described in Yusuf et al. [22]. Carbohydrates were detected by the anthrone test [23], and for reducing sugars, by the Fehling's test (https://en.wikipedia.org/wiki/Fehling%27s_solution) [24].

Polysaccharide content

Polysaccharide content was determined using the modified phenol-sulfur acid method of Dubois et al. [25]: 5.0 ml of concentrated water extract was combined with 20.0 ml of 87.5% ethanol, incubated overnight at 4°C, and centrifuged at 7000 rpm for 30 min. The sediment was washed twice with absolute ethanol, dried in vacuum, and dissolved in distilled water. The reaction was initiated by mixing 1 ml of polysaccharide solution with 0.5 ml 5% phenol and 2.5 ml concentrated sulfuric acid, incubated in a water bath at 100°C for 15 min, and cooled to room temperature. Optical density was determined at the wavelength of 490 nm; concentration of polysaccharides was calculated using a calibration curve built for D-glucose. Results were expressed as µg of glucose equivalent per 1 mg of extract.

Phenol content

Total content of phenols in water and ethanol extracts was determined according to the Folin-Ciocalteu colorimetric method with some modifications [26]: 0.5 ml of solution was mixed with 0.5 ml of Folin-Ciocalteu reagent (Sigma, USA) and incubated for 3 min. Then we added 0.5 ml of 20% sodium carbonate, adjusted the mixture to 5 ml with distilled water, and incubated in the dark for 90 min. Optical density was determined at the wavelength of 725 nm; concentration of phenols was calculated using a calibration

curve built for gallic acid. Results were expressed as μg of gallic acid equivalent per 1 mg of extract.

Flavonoid content

Total flavonoid content was determined according to Park et al. [27]: 1.0 ml of water-ethanol extract solution containing about 1.0 mg/ml of dried substance was mixed with 4.3 ml of solution containing 4.1 ml of 80% ethanol, 0.1 ml of 10% aqueous solution of $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and 1.0 ml of 1M aqueous potassium acetate ($\text{CH}_3\text{CO}_2\text{K}$). Reaction mixture was incubated for 40 min at room temperature. Optical density was measured at the wavelength of 415 nm. Flavonoid content was determined based on the calibration curve built using quercetin; results were expressed in μg of quercetin equivalent per 1 mg of extract.

Fatty acids content

Fatty acids were detected in hexane and ethyl acetate extracts as methyl esters, obtained by treatment with hydrogen chloride [28]. Separation was performed by gas chromatography–mass spectrometry (GC-MS) on an Agilent 7890N/5975C device (Agilent Technologies, USA) with a quadrupole 5973N mass spectrometer (Agilent Technologies, USA) and a DB-1 capillary column. Both total ion current in the 10–800 Da range and selected ion monitoring analysis were performed. Methyl esters of fatty acids were identified using the NIST Mass Spectral Search Program for the NIST / EPA / NIH Mass Spectral Library Version 2.0a. Quantitation was performed using the internal 1 $\mu\text{g}/\text{ml}$ tetrafluorobenzobarene standard.

Results and Discussion

We analyzed nine specimens of *G. lucidum* from various regions of Altai (Table 1). Fragments of the nuclear ribosomal spacer about 300 bp long containing the ITS1 spacer were sequenced and aligned; all nine sequences turned out to be identical. We constructed a phylogenetic tree based on the sequences listed in Table 1; the tree from Hseu et al. was used as a reference [14]. Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random

amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences [14], and *Gastrum pectinatum* (EU784241) was taken as the out-group.

The results of phylogenetic analysis are shown on Figure 1. Altai samples of *G. lucidum* were clearly distinct from other specimens. In addition to *G. lucidum*, the group containing Altai reishi also included *G. oerstedii*, *G. tsugae*, *G. valesiacum*, *G. oregonense*, *G. ahmadii*, and *G. carnosum*. According to Hseu et al. [14] this is group I of the *G. lucidum* complex, which is widespread in Argentina, Canada, USA, Pakistan, Britain, France, Norway, and Western Siberia.

Therefore, we found that all studied Altai specimens of *G. lucidum* have identical ITS1 sequences and belong to group I of this species. Our results accord with the data of Zhang et al., who also found that four reishi samples from Altai belong to this group [13].

Chemical composition of reishi fruit bodies

Water content in reishi fruit bodies was 10.78% and 11.47% in two experimental replicates; ash content, 6.01% and 6.27%. Hexane extracts (G) yielded 71 and 77 mg, respectively (an average of 0.66% of the absolutely dry substance); ethyl acetate extracts (EA+), 816 and 864 mg (3.78%); ethanol extracts (EtOH+), 1269 and 1332 mg (5.86%); water extracts (W+), 1277 and 1422 mg (6.07%). Under stepwise extraction, EA- extracts yielded 346 and 376 mg in two experimental replicates (an average of 3.22% of the absolutely dry substance); EtOH-, 251 and 293 (2.43%); W-, 602 and 618 mg (5.44%). The results of the phytochemical screening are shown in Table 2.

Results of the screening suggest that ethanol fractions contain mostly flavonoids, phenols, and coumarins; water fractions are dominated by tannins, carbohydrates, and coumarins; and hexane and ethyl acetate extracts, by terpenoids.

Fatty acid composition of the EA+ extract is given in Table 3. The main fatty acids were palmitic (7.37%), oleic and linoleic (41.40 %), and linolenic (13.95%) acids, as well as traces of myristic, pentadecanoic, palmitoleic, and stearic acids, which conforms to

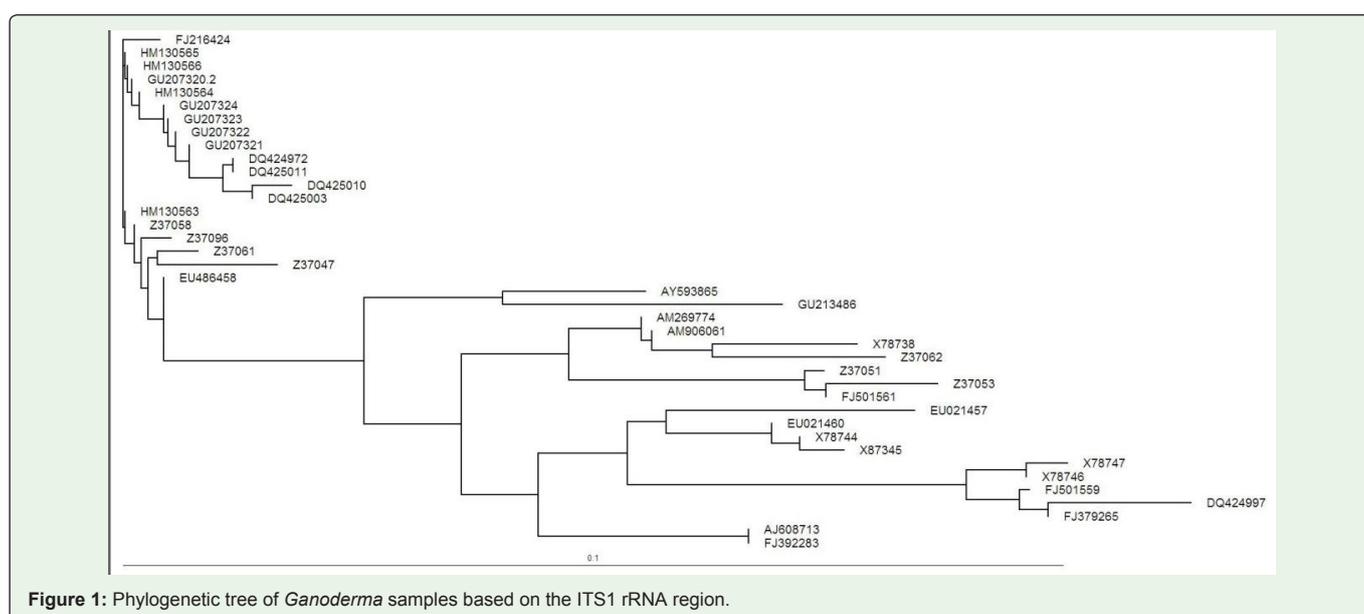


Figure 1: Phylogenetic tree of *Ganoderma* samples based on the ITS1 rRNA region.

Table 1: Specimens used in this study.

Group ^a	Species	Origin	GenBank no.	Source
I	<i>G. lucidum</i>	Altai	GU207321	This study
I	<i>G. lucidum</i>	Altai	GU207322	This study
I	<i>G. lucidum</i>	Altai	GU207323	This study
I	<i>G. lucidum</i>	Altai	HM130564	This study
I	<i>G. lucidum</i>	Altai	GU207324	This study
I	<i>G. lucidum</i>	Altai	HM130565	This study
I	<i>G. lucidum</i>	Altai	GU207320.2	This study
I	<i>G. lucidum</i>	Altai	HM130563	This study
I	<i>G. lucidum</i>	Altai	HM130566	This study
I	<i>G. ahmadii</i>	Pakistan	Z37047	[14]
I	<i>G. oregonense</i>	Canada	Z37061	[14]
I	<i>G. lucidum</i>	Norway	Z37096	[14]
I	<i>G. oerstedii</i>	Argentina	Z37058	[14]
II.1	<i>G. lucidum</i>	Taiwan	X87345	[14]
II.1	<i>G. lucidum</i>	India	X78744	[14]
II.2	<i>G. lucidum</i>	Japan	X78746	[14]
II.2	<i>G. lucidum</i>	Taiwan	X78747	[14]
III.1	<i>G. lucidum</i>	United States	Z37051	[14]
III.1	<i>G. lucidum</i>	United States	Z37053	[14]
III.2	<i>G. resinaceum</i>	United Kingdom	Z37062	[14]
III.2	<i>G. pfeifferi</i>	Netherlands	X78738	[14]
	<i>G. japonicum</i>	-	AY593865	[29]
	<i>G. mastoporum</i>	-	GU213486	Huang L.H., Wu Q.P., Yang X.B.
	<i>G. pseudoferreum</i>	-	FJ392283	Zhang X., Xie Y., Qi Y., Pu J., Zhang H.
	<i>G. philippii</i>	-	AJ608713	Bougher N.L.
I	<i>G. carnosum</i>	-	EU486458	Denis M.W., Berbee M.L.
	<i>G. tropicum</i>	-	EU021457	Wang D.-M., Wu, S.-H
I	<i>G. lucidum</i>	China	DQ424972	Su C.L., Zhang J.S., Pan Y.J.
I	<i>G. lucidum</i>	China	DQ425010	Su C.L., Zhang J.S., Pan, Y.J.
I	<i>G. oerstedii</i>	China	DQ425011	Su C.L., Zhang J.S., Pan, Y.J.
I	<i>G. lucidum</i>	-	FJ216424	Gao X.X., Yang L.
I	<i>G. tsugae</i>	China	DQ425003	Su C.L., Zhang J.S., Pan, Y.J.
III.2	<i>G. pfeifferi</i>	Italy	AM269774	[30]
III.2	<i>G. resinaceum</i>	Italy	AM906061	[30]
III.1	<i>G. lucidum</i>	-	FJ501561	Jia D.H., Peng W.H., Gan B.C.
II.1	<i>G. lucidum</i>	-	EU021460	Wang D.-M., Wu S.-H.
II.2	<i>G. lucidum</i>	-	FJ501559	Jia D.H., Peng W.H., Gan B.C.
II.2	<i>G. lucidum</i>	China	DQ424997	Su C.L., Zhang J.S., Pan Y.J.
II.2	<i>G. lucidum</i>	-	FJ379265	Huang L.H., Wu Q.P., Yang X.B.

Table 2: Results of the phytochemical screening.

Fraction	Alkaloids	Flavonoids	Terpenoids	Tannins	Saponins	(Poly)Phenols	Coumarins	Carbohydrates	Reducing sugars
G	-	-	++	-	-	-	-	-	-
EA-	-	+	++	-	-	+	-	-	-
EtOH-	+	++	+	+	-	++	++	-	-
W-	-	+	-	++	+	+	++	++	+
EA+	-	+	++	-	-	+	-	-	-
EtOH+	+	++	+	+	-	++	++	-	-
W+	-	+	-	++	+	+	++	++	+

Extracts: G, Hexane; EA-: Ethyl Acetate after G; EtOH-: Ethanol after EA; W-: Water after EtOH-; EA+: Ethyl Acetate; EtOH+: Ethanol; W+: Water.

Table 3: Fatty acids in the ethyl acetate extract.

Peak number	Elution time, min	Content, %	Acid type
Peak 1	10,521	0.35	C _{14:0} Miristic
Peak 2	10,992	1.35	C _{15:0}
Peak 2	11,338	0.64	C _{16:1(9E)} Palmitoleic
Peak 2	11,572	7.37	C _{16:0} Palmitic
Peak 3	12,248	41.40	C _{18:1(9Z)} + C _{18:2ω6} Oleic + Linoleic
Peak 5	12,440	13.95	C _{18:3} Linolenic
Peak 6	13,139	2.12	C _{18:0} Stearic

the composition of *G. lucidum* from China [31] also found 189.1-1453.3 µg/g of ergosterol in *G. lucidum*.

Ergosterol isolated from the hexane extract had melting point at 160-162°, which accords with the value of 160° determined by Pruess et al., *litas.* 161°C [32]; its spectral characteristics were identical to those taken from the Biological Magnetic Resonance Data Bank (bmse000494) [33], and it had no melting point depression with a known sample. In an earlier study we found 7.17% ergosterol in methanol extracts of *G. lucidum* (Shevelev OB, Seryapina AA, Goryachkovskaya TN, Tatarova LE, Slyngo NM, Peltek SE, Markel AL, Moshkin MP Hypotensive and Neurometabolic Effects of Intra-gastric Reishi (*Ganoderma lucidum*) Administration in Hypertensive Rat Strain article in preparation).

W+ and W- extracts were used to determine protein concentration using the Quick Start™ Bradford Protein Assay Kit 4 (Bio-Rad, CIIIA). In two experimental replicates we found protein concentration of 202 and 203 µg/ml for W+, and 103 and 101 µg/ml for W-, which corresponds to 2.03 and 1.02 mg/g of soluble proteins, respectively. Twofold reduction of protein concentration in W- is probably correlated with partial insolubilization of proteins during ethanol treatment.

According to published studies, ethyl acetate and ethanol extracts contained mostly phenols, flavonoids, and triterpenic acids [34]. We found that fruit bodies of Altai *G. lucidum* contained 32.4 mg of phenols per 1 g of extract (in pyrocatechol equivalent), while flavonoids made up 11.1 mg per g (in quercetin equivalent).

Polysaccharide content in water extract of the sediment was 752 mg/g after extraction by organic solvents, as determined by the phenol-sulfur acid method, which corresponds to 4.55% of the absolutely dry substance. Extraction of chopped fruit bodies with hot

water after concentration and drying yields 2.66 g of dry sediment (10.6% of the absolutely dry substance), and polysaccharides make up 735 mg/g of this extract (calculated based on glucose), which corresponds to 4.44% of the absolutely dry substance.

However, certain studies suggest that BAS yield can be substantially increased using aqueous alkaline solution, which facilitates the extraction of acid polysaccharides [21]. We performed this procedure for the insoluble sediment formed after stepwise extraction and dialysis. Polysaccharide yield was 3.8 mg per ml of concentrated dialyzate, which corresponds to 6.16% of dried insoluble sediment after water extraction. The final quantity of polysaccharides that we managed to extract from reishi fruit bodies was 10.72% of the absolutely dry substance. On the whole, chemical composition of reishi from Altai is similar to that from other regions.

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