Phytochemistry 127 (2016) 29–37

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Distinguishing commercially grown *Ganoderma lucidum* from *Ganoderma lingzhi* from Europe and East Asia on the basis of morphology, molecular phylogeny, and triterpenic acid profiles

Florian Hennicke [a,b,1], Zakaria Cheikh-Ali [c,1], Tim Liebisch [a], Jose G. Maciá-Vicente [a], Helge B. Bode [c,d], Meike Piepenbring [a,*]

[a] Department of Mycology, Cluster for Integrative Fungal Research (IPF), Institute of Ecology, Evolution, and Diversity, Goethe Universität Frankfurt, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany
[b] Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25, 60325 Frankfurt am Main, Germany
[c] Merck Stiftungsprofessur für Molekulare Biotechnologie, Fachbereich Biowissenschaften, Goethe Universität Frankfurt, 60438 Frankfurt am Main, Germany
[d] Buchmann Institute for Molecular Life Sciences (BMLS), Goethe Universität Frankfurt, 60438 Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 15 December 2015
Received in revised form 22 February 2016
Accepted 23 March 2016
Available online 1 April 2016

Keywords:
Ganoderma lucidum
Ganoderma lingzhi
Ganodermataceae
Polyporales
Fungi
Ling-zhi
Lingzhi
Reishi
Triterpenic acids
Ganoderic acids

ABSTRACT

In China and other countries of East Asia, so-called Ling-zhi or Reishi mushrooms are used in traditional medicine since several centuries. Although the common practice to apply the originally European name '*Ganoderma lucidum*' to these fungi has been questioned by several taxonomists, this is still generally done in recent publications and with commercially cultivated strains. In the present study, two commercially sold strains of '*G. lucidum*', M9720 and M9724 from the company Mycelia bvba (Belgium), are compared for their fruiting body (basidiocarp) morphology combined with molecular phylogenetic analyses, and for their secondary metabolite profile employing an ultra-performance liquid chromatography–electrospray ionization mass spectrometry (UPLC–ESIMS) in combination with a high resolution electrospray ionization mass spectrometry (HR-ESI-MS). According to basidiocarp morphology, the strain M9720 was identified as *G. lucidum* s.str. whereas M9724 was determined as *Ganoderma lingzhi*. In molecular phylogenetic analyses, the M9720 ITS and beta-tubulin sequences grouped with sequences of *G. lucidum* s.str. from Europe whereas those from M9724 clustered with sequences of *G. lingzhi* from East Asia. We show that an ethanol extract of ground basidiocarps from *G. lucidum* (M9720) contains much less triterpenic acids than found in the extract of *G. lingzhi* (M9724). The high amount of triterpenic acids accounts for the bitter taste of the basidiocarps of *G. lingzhi* (M9724) and of its ethanol extract. Apparently, triterpenic acids of *G. lucidum* s.str. are analyzed here for the first time. These results demonstrate the importance of taxonomy for commercial use of fungi.

1. Introduction

Fungi produce a high diversity of low molecular weight natural products with different bioactivities that often are important for their interactions with other organisms (Brakhage and Schroeckh, 2011). In the case of so-called Ling-zhi or Reishi fungi, an impressively large arsenal of more than 400 identified bioactive compounds is produced (Chen et al., 2012). These fungi have a long history of more than 2000 years (Anonymous, 1955) of being used as the mushroom of immortality (Pegler, 2002) in traditional medicine in China, Japan, Taiwan, and other Asian regions (Shiao, 2003). Ling-zhi products made from cultivated Ling-zhi fungi are consumed worldwide and have a significant market value (Wachtel-Galor et al., 2004), e.g., an estimated annual global turnover of more than 2.5 billion US dollars according to Li et al. (2013). Modern research has shown that Ling-zhi fungi have various biological activities and medicinal properties, such as anticancer, antihypertensive, antiviral, and immunomodulatory activities (Boh et al., 2007). These activities are mainly due to polysaccharides and triterpenoids that are highly oxygenated lanosterol derivatives known as triterpenic acids (ganoderic acids), ganoderiols, ganolucidic acids, lucidones, and lucidenic acids. They differ from each other by their oxidation states (Cole et al., 2003).

* Corresponding author.
E-mail addresses: hennicke@em.uni-frankfurt.de (F. Hennicke), Cheikhali@bio.uni-frankfurt.de (Z. Cheikh-Ali), tim.liebisch@stud.uni-frankfurt.de (T. Liebisch), MaciaVicente@em.uni-frankfurt.de (J.G. Maciá-Vicente), h.bode@bio.uni-frankfurt.de (H.B. Bode), piepenbring@em.uni-frankfurt.de (M. Piepenbring).
[1] These authors contributed equally to this manuscript.

http://dx.doi.org/10.1016/j.phytochem.2016.03.012
0031-9422/

The species *Ganoderma lucidum* (Curtis) P. Karst. (Ganodermataceae, Polyporales, Basidiomycota) was originally described as *Boletus lucidus* Curtis based on specimens found in England (Curtis, 1781). Later, the name *G. lucidum* was erroneously applied to morphologically somewhat similar *Ganoderma* collections with laccate pilei from many countries all over the world, including the Ling-zhi fungus in East Asia. Although about 20 years ago it was shown that specimens named *G. lucidum* from Europe and East Asia mostly are not conspecific (Moncalvo et al., 1995a,b), Ling-zhi isolates employed in medicinal, chemical, or genomic studies as well as commercially cultivated strains are still referred to as *G. lucidum* (Baby et al., 2015; Chen et al., 2012; Keypour et al., 2010; Wang et al., 2012).

Meanwhile, morphological characteristics helpful to distinguish the European *G. lucidum* s.str. and the Ling-zhi fungus are known and several taxonomic studies are available (Cao et al., 2012; Wang et al., 2009, 2012; Yao et al., 2013; Zhou et al., 2015). It is still unclear, however, which name based on a Ling-zhi type specimen has priority and should be applied. As long as there is no world monograph including a detailed investigation of type specimens of the approximately 290 taxonomic names referring to maybe 80 species of *Ganoderma* (Kirk et al., 2008; Ryvarden, 2000), it will not be possible to apply a taxonomically correct name with certainty. An old name based on a Ling-zhi type specimen may exist, but it is difficult or even impossible to prove that because many type specimens are lost or in a bad state. Therefore, in the present manuscript we use the name *G. lingzhi* Sheng H. Wu, Y. Cao & Y.C. Dai for the Ling-zhi fungus, which is evidenced by morphological and molecular data (Cao et al., 2012; Hawksworth, 2013; Yang and Feng, 2013).

In the publications mentioned above, authors focused on morphology and taxonomy based on morphological characteristics and molecular sequence data. They did not investigate corresponding secondary metabolite profiles such as the content of triterpenic acids in these fungi that could potentially be useful for chemotaxonomy (Baby et al., 2015). These triterpenic acids are the cause for the bitter flavour of the basidiocarps of certain *Ganoderma* spp. (Kubota et al., 1985; Nishitoba et al., 1985). For the Ling-zhi fungus (probably *Ganoderma lingzhi*, cited as '*G. lucidum*'), Yang et al. (2007) identified 32 and Cheng et al. (2010) 43 triterpenoids. In total, over 240 secondary compounds with triterpenoids being major constituents were isolated from the Ling-zhi fungus, and over 431 from *Ganoderma* spp. according to Baby et al. (2015). Although the authors cited above are aware of the taxonomic confusion, they still cite '*G. lucidum*' as name for the Ling-zhi fungus.

In the present study, we analyzed two commercially cultivated '*G. lucidum*' strains, i.e., M9720 and M9724, from a professional spawn and mycelium culture supplier in Belgium (Mycelia bvba) (Fig. 1). For the first time, here, a combined approach was used, including morphological characteristics of the basidiocarps, molecular phylogenetic analyses, and triterpenic acid profiling.

2. Results and discussion

2.1. Morphology

The macro- and micromorphological analysis of the basidiocarps of M9720 and M9724 yielded data on distinctive characteristics showing that M9720 represents *Ganoderma lucidum* s.str. and M9724 *Ganoderma lingzhi*. Distinctive characteristics published in literature mainly by Cao et al. (2012), Yao et al. (2013), and Zhou et al. (2015) are complemented by own observations (Fig. 2 and Table 1).

At the start of the present project, mushroom cultivators noticed that the basidiocarp powder and the alcoholic extract of basidiocarps of M9724 was bitter, while the extract of M9720 was not (pers. com. E. Noll). This aspect was checked by the authors by placing a piece (ca. $1 \times 1 \times 0.5$ cm^3) of dry basidiocarp onto the basal part of the tongue after some chewing: A strong bitter taste is evident only for M9724, i.e., *G. lingzhi*.

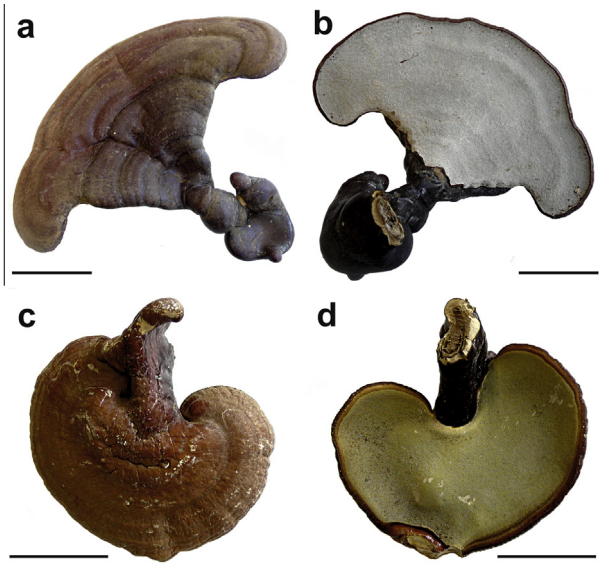

Fig. 1. (a-b) A basidiocarp of *Ganoderma lucidum* (M9720). (a) Pileal surface and stipe. (b) Pore surface and stipe. (c-d) A basidiocarp of *Ganoderma lingzhi* (M9724). (c) Pileal surface and stipe. (d) Pore surface and stipe. Note the yellow colour of the hymenophore. Both fungi were cultivated in similar conditions in the Pilzfarm Noll. Photos were taken in natural light. Scale bars = 3 cm.

Pores of *G. lucidum* (M9720) (Fig. 2a) have a more regular circular to ovoid outline and fuse more often with neighbouring pores than in hymenophores of *G. lingzhi* (M9724) (Fig. 2b).

Further distinctive characteristics were observed for basidiospores that, as seen by light microscopy, present an outer, hyaline layer called myxosporium and a brown coloured exosporium carrying spines that protrude into the myxosporium (comp. Fig. 2c; Mims and Seabury, 1989). The myxosporium is interrupted at the hilum and the spores are usually truncate at the apex.

While the length of basidiospores measured including the myxosporium is rather similar for *G. lucidum* (M9720) and *G. lingzhi* (M9724) (Figs. 2d, e and 3a), basidiospores were significantly ($W = 457$, $P < 0.001$) shorter in *G. lingzhi* (M9724) than in *G. lucidum* (M9720) when measured without the myxosporium (Figs. 2d, e and 3b). This is due to the fact that the myxosporium protrudes at the tip of basidiospores of *G. lingzhi* (M9724) by approximately 1 µm, while it is almost completely perforated by spines of the exosporium in basidiospores of *G. lucidum* (M9720). The width of basidiospores is significantly smaller in *G. lingzhi* (M9724) than in *G. lucidum* (M9720) both when measurements include or not the myxosporium ($W = 573$, $P < 0.001$ and $W = 541$, $P < 0.001$, respectively; Fig. 3).

2.2. Molecular phylogenetic analyses

The phylogenetic analyses based on the ITS and *β-tub* sequences of M9720 and M9724 and of a selection of reference sequences from *Ganoderma* species confirm previous results which relocate specimens originally identified as *G. lucidum* s.lat. into three separate species, namely *G. lingzhi*, *G. lucidum* s.str., and *Ganoderma multipileum* (Fig. 4 and Table S1; Cao et al., 2012). Moreover, in the analysis of the *β-tub* gene sequences, *G. lucidum* s.str.

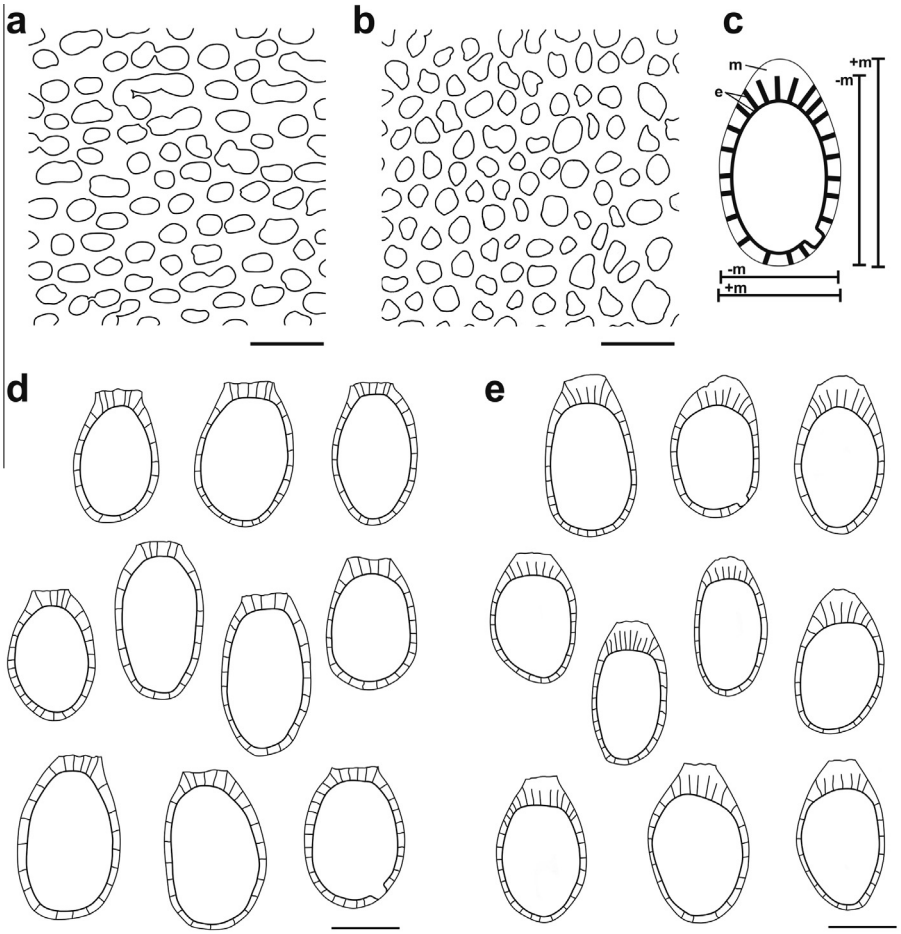

Fig. 2. Morphology of *Ganoderma lucidum* (M9720) and *Ganoderma lingzhi* (M9724). (a-b) The openings of the pores of two basidiocarps as seen with a stereomicroscope. Scale bars = 0.5 mm. (a) *G. lucidum* s.str. (b) *G. lingzhi*. (c) Schematic illustration of a basidiospore of *Ganoderma* sp. See from outside to inside the myxosporium (hyaline, m), the exosporium (brown, e) carrying spines that protrude into the myxosporium, and the hilum that interrupts these layers. Bars show positions of measurements with (+m) and without (−m) the myxosporium. (d-e) Basidiospores as seen by light microscopy. Scale bars = 5 µm. (d) *G. lucidum* s.str. (e) *G. lingzhi*.

Table 1
Distinctive characteristics of *Ganoderma lucidum* s.str. and *Ganoderma lingzhi* based on own observations of the specimens *G. lucidum* (M9720) and *G. lingzhi* (M9724), as well as on data cited from literature.

Characteristics	*Ganoderma lucidum*	*Ganoderma lingzhi*
Taste	–	Bitter
Pore surface, colour at maturity	White, grey to brownish when old	Sulphur yellow, straw-coloured when dry
Fusion of pores with adjacent pores	Often	Rare
Basidiospores, length including the myxosporium	(9–)10–11.5(–12) µm 9–10.5(–11) µm[a]	(9–)10–11.5(–12) µm (9–)9.5–11(–13) µm[a] (8–)9–11.5(–12.5) µm[b]
Basidiospores, width including the myxosporium	(5–)6–7.5(–8) µm 5.5–6.5(–7) µm[a]	(5–)5.5–6.5(–7) µm (5–)5.5–7(–8) µm[a] (5.5–)6–8(–8.5) µm[b]
Basidiospores, length without the myxosporium	8.5–9.5(–10) µm (9–)9.5–12(–13) µm[c]	7–8.5(–10) µm (8–)9–10.5(–12) µm[a,c] (5–)6.5–8.5(–9.5) µm[b]
Basidiospores, width without the myxosporium	(5–)5.5–6.5(–7) µm 6–8(–8.5) µm[c]	5–5.5(–6) µm (5–)6–7(–7.5) µm[a,c] 4.5–6.5(–7) µm[b]

[a] Cao et al. (2012; values rounded).
[b] Yao et al. (2013, with *G. lingzhi* being cited as *G. sichuanense*).
[c] Zhou et al. (2015).

specimens with a Eurasian origin are located in a clade distant from the clade including sequences from the North-American species *Ganoderma tsugae* and *Ganoderma oregonense*, which appear closely related to *G. lucidum* s.str. in ITS-based phylogenies (Fig. 4; Cao et al., 2012; Zhou et al., 2015). Based on our results, we propose new identifications for several strains of *Ganoderma* spp., the names of which were in conflict with their position in the phylogenetic trees (Fig. 4 and Table S1).

Both the ITS and the *β-tub* phylogenetic reconstructions place strains M9720 and M9724 in independent clades (Fig. 4). M9720

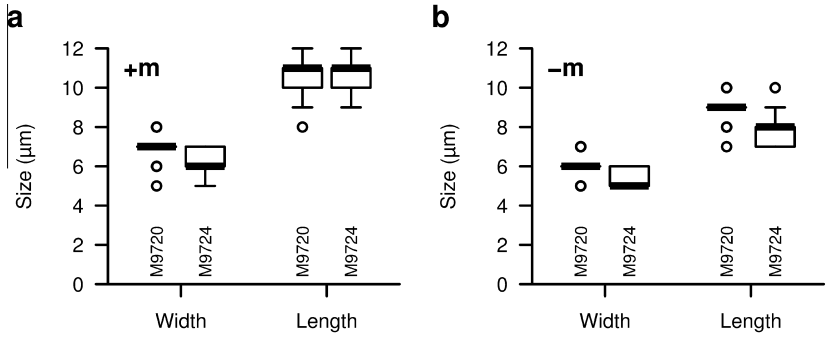

Fig. 3. Values of width and length of basidiospores of *Ganoderma lucidum* (M9720) and *Ganoderma lingzhi* (M9724). (a) Including the myxosporium (+m). (b) Measured without the myxosporium (−m).

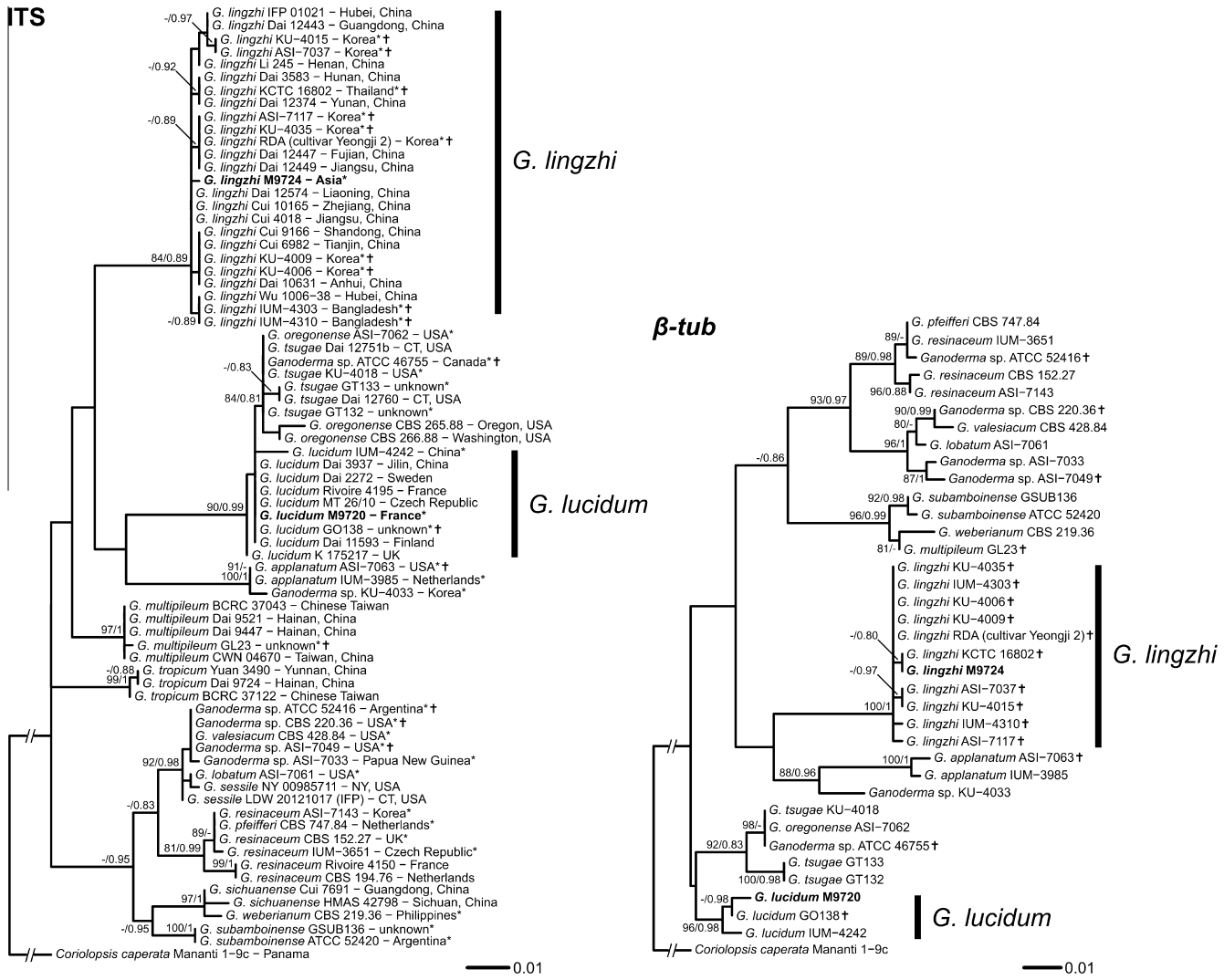

Fig. 4. Placement of sequences of the ITS region and the β-tubulin (*β-tub*) gene of the strains M9720 and M9724 (both in bold) among sequences of *Ganoderma* spp. (Table S1) using phylogenetic reconstructions. Both trees represent maximum likelihood (ML) topologies. ML bootstrap and posterior probability (PP) support values are displayed next to each node (ML/PP). In the ITS tree, geographical origin of the specimens is shown, and asterisks denote sequences based on specimens also represented in the *β-tub* phylogeny. Some names available in GenBank (Table S1) were amended according to the data presented here and marked by cross marks.

is clustered alongside *G. lucidum* s.str. strains predominantly with a Western European origin. On the other hand, strain M9724 is placed in a strongly supported clade containing specimens from East Asia, which have been attributed to the species *G. lingzhi* (Cao et al., 2012).

2.3. Identification of triterpenoids

The ethanol extracts of ground basidiocarp powder of strains M9720 and M9724 were analyzed for triterpenic acids (ganoderic acids) by HPLC coupled with mass spectrometry. Overall, M9720

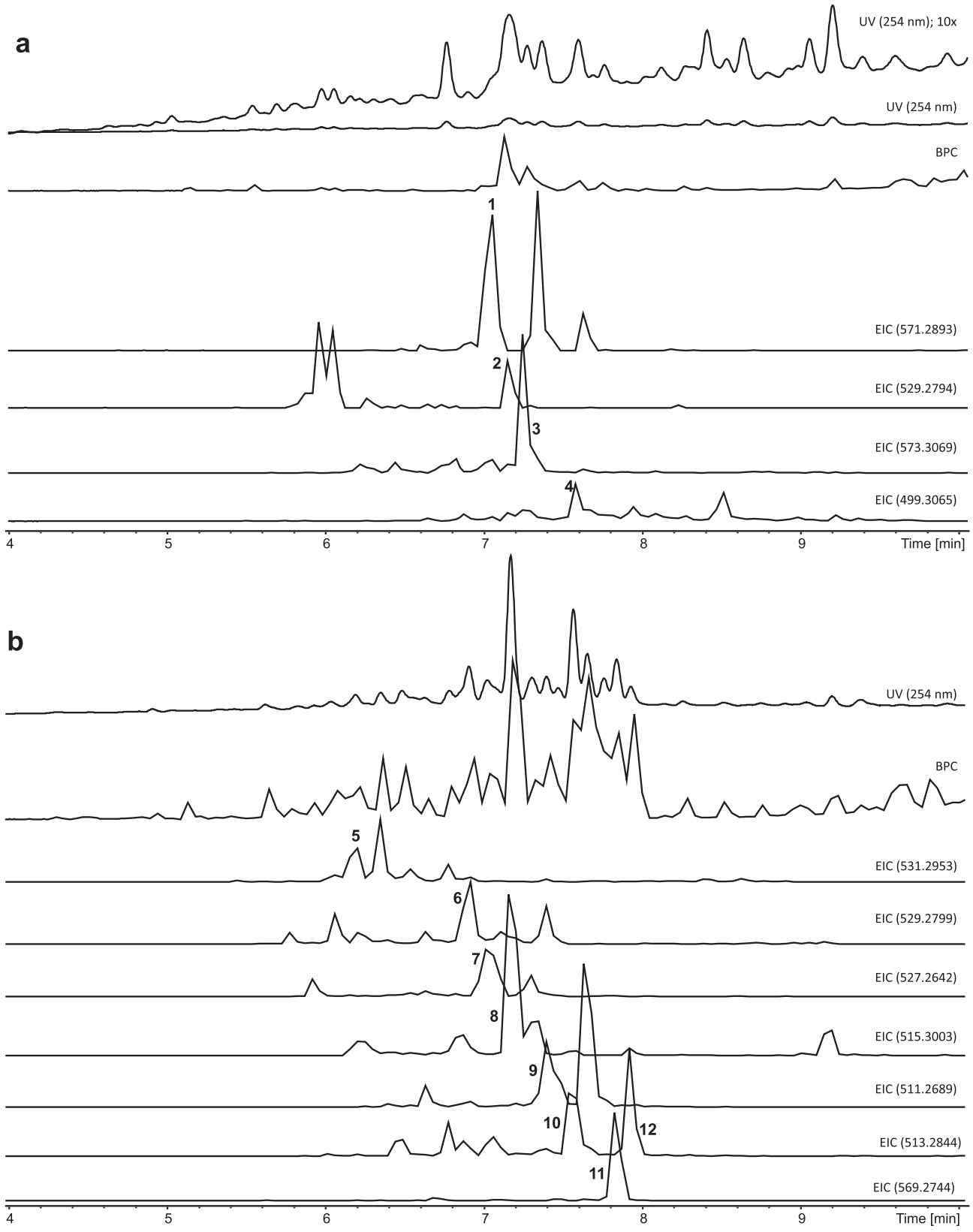

Fig. 5. HPLC/MS analyses of ethanol extracts from basidiocarps of *Ganoderma lucidum* (M9720, a) and *Ganoderma lingzhi* (M9724, b). From top to bottom UV (254 nm), base peak chromatograms (BPC, negative polarity), and extracted ion chromatograms (EIC, see Table 2) of identified triterpenoids are shown for both extracts. UV and BPC in (a) and (b) are drawn to the same scale. For better visualization of signals in M9720 (a) an additional 10-fold amplification is shown.

contained much less triterpenic acid than M9724 as can be seen from the UV chromatograms of both strains drawn to the same scale (Fig. 5a and b). This is in agreement with its lack of a bitter taste compared to M9724 as described above. Triterpenic acids were identified by high-resolution masses and ESI-MS^2 fragmentation ions using a Q-ToF (Impact II, Bruker). Negative mode ESI/MS

Table 2
Triterpenic acids identified from basidiocarps of *Ganoderma* spp.

No	Metabolite	R_t (min)	Sum formula [M]	*m/z Calc.* $[M-H]^-$	*m/z det.* $[M-H]^-$	Δ ppm	-MS/MS (*m/z*) fragmentation
Ganoderma lucidum (M9720)							
1	Ganoderenic acid K	6.9	$C_{32}H_{44}O_9$	571.2913	571.2893	3.5	553, 538, 529, 511, 494, 467, 450, 435, 303, 265
2	Ganoderic acid C6	7.1	$C_{30}H_{42}O_8$	529.2807	529.2794	2.5	511, 481, 437
3	Ganoderic acid K	7.2	$C_{32}H_{46}O_9$	573.3069	573.3053	2.8	555, 511, 496, 453, 267
4	Ganolucidic acid A	7.5	$C_{30}H_{44}O_6$	499.3065	499.3047	2.8	481, 469, 455, 273
Ganoderma lingzhi (M9724)							
5	Ganoderic acid G	6.2	$C_{30}H_{44}O_8$	531.2963	531.2953	1.9	513, 498, 469, 452, 437, 336, 302
6	12-Hydroxy-ganoderic acid D	6.9	$C_{30}H_{42}O_8$	529.2807	529.2799	1.5	511, 494, 467, 449, 301
7	Elfvingic acid A	7.1	$C_{30}H_{40}O_8$	527.2650	527.2642	1.5	509, 479, 465, 435
8	Ganoderic acid B	7.2	$C_{30}H_{44}O_7$	515.3014	515.3003	2.1	497, 453, 439, 393, 304, 288, 250
9	Ganoderenic acid D	7.4	$C_{30}H_{40}O_7$	511.2701	511.2689	2.3	493, 449, 434, 301, 285
10	Ganoderic acid AM1	7.6	$C_{30}H_{42}O_7$	513.2858	513.2844	2.7	495, 451, 436, 249
11	12-Acetoxy-ganoderic acid F	7.8	$C_{32}H_{42}O_9$	569.2751	569.2744	1.2	551, 507, 487, 465, 435, 301
12	Ganoderic acid J	7.9	$C_{30}H_{42}O_7$	513.2858	513.2844	2.7	451, 437, 422, 301, 285

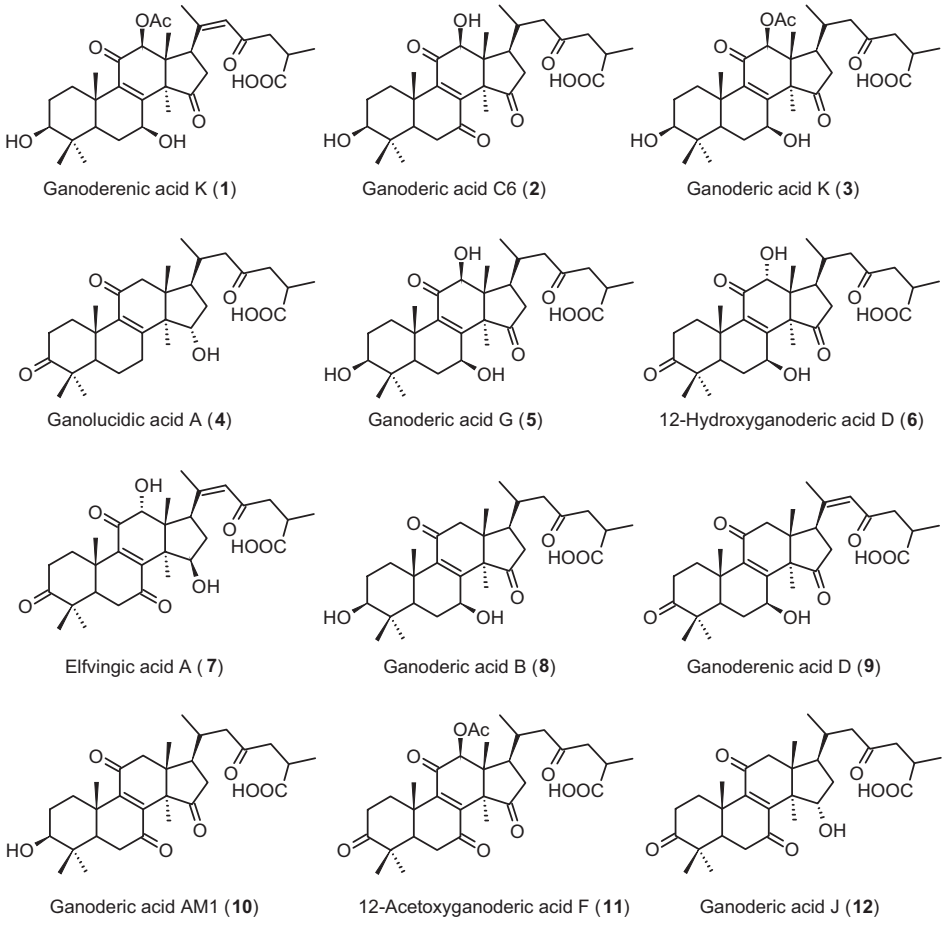

Fig. 6. Triterpenic acids isolated and identified from *Ganoderma lucidum* s.str. (strain M9720; **1**–**4**) or *Ganoderma lingzhi* (strain M9724; **5**–**12**).

was used because of its sensitivity for these acids as previously described (Cheng et al., 2012, 2013; Yang et al., 2007). All triterpenoids gave $[M-H]^-$ and sometimes $[2M-H]^-$ ions by electrospray ionization monitored in the negative ion mode (Figs. S1–S12). Due to the large number of different triterpenoids from *Ganoderma* spp. with >350 known derivatives, not all compounds could be identified as several of them share the same mass and show only subtle retention time differences (Yang et al., 2007). However, from HR-MS and MS–MS data as well as known retention time differences (Yang et al., 2007; Baby et al., 2015; and references therein) overall 12 compounds have been identified in the two strains.

From the basidiocarps of strain M9720, we were able to identify four ganoderic acids (**1**–**4**), i.e., ganoderenic acid K (**1**), ganoderic acid C6 (**2**), ganoderic acid K (**3**), and ganolucidic acid A (**4**) (Table 2, Figs. 5, 6 and S1–S4). Eight ganoderic acids (**5**–**12**) were detected from the basidiocarps of M9724, i.e., ganoderic acid G (**5**), 12-hydroxyganoderic acid D (**6**), elfvingic acid A (**7**), ganoderic acid B (**8**), ganoderenic acid D (**9**), ganoderic acid AM1 (**10**), 12-acetoxyganoderic acid F (**11**), and ganoderic acid J (**12**), with ganoderic acid B (**8**) being the main derivative (Table 2, Figs. 5, 6 and S5–S12).

The dominant fragments corresponding to losses of H_2O and CO_2 are detected for all identified structures (Yang et al., 2007). Ganoderenic acid K (**1**, *m/z* 571.2893), for example, yielded

prominent ions at 571.4, 553.3, 511.3, and 467.3 *m*/*z* corresponding to $[M-H]^-$, $[M-H_2O-H]^-$, $[M-H_2O-CO_2-H]^-$, and $[M-OAc-CO_2-H]^-$ respectively. Ganoderic acid B (**8**, 515.3003) showed ions at 497.1 and 453.1 corresponding to $[M-H_2O-H]^-$ and $[M-H_2O-CO_2-H]^-$ (Table 2).

The compounds identified here for the *G. lingzhi* strain M9724 form part of the lists of identified triterpenoids published by Yang et al. (2007) and Cheng et al. (2010). In both papers, the studied organism is called '*G. lucidum*', but the authors refer to the medicinally used Ling-zhi fungus, i.e., most likely *G. lingzhi*. This conclusion is based on the assumption that triterpenoid profiles are specific for different species of *Ganoderma*, as demonstrated, e.g., by Zhao et al. (2006). These authors isolated significantly different compound spectra from *G. lingzhi* (cited as *G. lucidum*) and *Ganoderma sinense* J.D. Zhao, L.W. Hsu & X.Q. Zhang, represented by several strains from different geographic origins each, with a good repeatability for the presence and the quantification of main compounds (Zhao et al., 2006).

3. Concluding remarks

Two investigated strains, M9720 and M9724, were sold as '*G. lucidum*', but based on basidiocarp morphology and phylogenetic analyses, M9720 is identified as *G. lucidum* s.str. whereas M9724 represents the species *G. lingzhi*. Furthermore, our ganoderic acid profiling showed a higher diversity and higher amounts of ganoderic acids in M9724 than in M9720 that probably are responsible for the bitter taste of the ethanol extract of M9724 basidiocarp powder. As the triterpenoid contents is rather low, basidiocarps of *G. lucidum* s.str. probably are not medicinal mushrooms. However, *Ganoderma* basidiocarps might also contain other bioactive compounds like polysaccharides (Boh et al., 2007; Ferreira et al., 2015 and citations therein).

More extensive ganoderic acid profiling of different *Ganoderma* spp. could clarify whether ganoderic acid profiles can be used as chemotaxonomic traits and complement *Ganoderma* species identification which up to now is based on basidiocarp morphology and molecular phylogenetic analyses only (comp. Baby et al., 2015; Richter et al., 2015; and citations therein).

As all published chemical analyses of Ling-zhi fungi called '*G. lucidum*' accessible to the authors revealed a high diversity of triterpenoids and referred to medicinal mushrooms, they probably dealt with specimens of *G. lingzhi*. In order to confirm this, voucher specimens that were deposited by authors of published studies, should be investigated. To the best of our knowledge, the European *G. lucidum* s.str. is analyzed here for natural products for the first time.

4. Experimental

4.1. Fungal material

The '*Ganoderma lucidum*' strains used in this study, M9720 and M9724, originate from a professional spawn and mycelium culture supplier, Mycelia bvba (Belgium). The strain M9720 was originally isolated from a *Ganoderma lucidum* basidiocarp in France (Dordogne, La Roque-Gageac, Le Village, from a decaying tree stump) in 1991. The strain M9724 is of uncertain origin. Mycelial cultures of M9720 and M9724 were maintained on potato dextrose agar (PDA) medium (AppliChem, Darmstadt, Germany) and kept as frozen stocks in 15% glycerol at −80 °C. Basidiocarps of M9720 and M9724 were grown from commercially produced mushroom spawn bags (Pilzzucht Hesse, Stadtallendorf, Germany). The spawn substrate consisted of 46.7% sawdust, 7.33% cereals, 2.33% oil seed, 0.33% chalk, and 43.33% water. The sawdust originated from untreated *Fagus sylvatica* wood (Brix Andrzej Wienke, Krystian Marcianiak GbR, Poland), contained 30–40% water, and was seasoned for at least three months previous to utilization as a spawn substrate ingredient. The content of sawdust particles smaller than 1 mm accounted for more than 90% of the total volume. The ingredients of the substrate were thoroughly blended, the spawn substrate was allotted at 2.6 kg aliquots into high-density polyethylene (HDPE) bags with a Tyvek™ filter (King Nonwovens B.V., The Netherlands), and then autoclaved at 111 °C for 10 h using a Condibox autoclave (Welker Spintech GmbH, Germany).

Spawn bags were inoculated using a 16 cm long and 0.4 cm diameter wooden skewer that was fully overgrown by M9720 or M9724 mycelium, respectively, and delivered to Pilzzucht Hesse by Mycelia bvba (Belgium). Inoculated spawn bags were incubated for 5–6 weeks at 22 °C in the dark.

Basidiocarps were produced from fully grown spawn bags at a mushroom farm (Pilzfarm Noll, Weinbach, Germany) by applying fruiting conditions (bags cut open, 80–90% atmospheric humidity, 25 °C, daylight). Mature basidiocarps were harvested after about six weeks. Maturity is reached when basidiocarp growth stops and basidiospores are massively shed and form a brown spore mass on the upper side of the basidiocarp. At the same time, the growing edge of the basidiocarp turns from white to brown (M9720) or yellow to brown (M9724), respectively.

Dry basidiocarps of the strains M9720 and M9724 are deposited in the Senckenberg Forschungsinstitut und Naturmuseum at Frankfurt am Main, Germany (M9720: FR-0247027, M9724: FR-0247026).

4.2. Characterization of basidiocarp morphology

Air-dried mature basidiocarps were used to investigate the basidiocarp morphology of M9720 and M9724. Pore surfaces were drawn as observed under a stereomicroscope (Nikon CP-5), while basidiospores were drawn and measured with maximum magnification (with oil objective 100×) of a light microscope (Olympus CX41RF). For each of the different aspects of basidospore size (length/width, including/without the myxosporium), values of 50 different spores were measured. The values were visualized by means of boxplots, and they were compared between specimens using the Wilcoxon rank-sum test as implemented in R v3.0.2 (R Core Team, 2013).

4.3. Phylogenetic analyses

For phylogenetic analysis, sequences of the internal transcribed spacers of the ribosomal DNA (ITS) and partial beta-tubulin gene (*β-tub*) were obtained for M9720 and M9724. Genomic DNA from both strains was obtained from fresh mycelial cultures grown on PDA for ten days at 25 °C, using the protocol described by O'Donnell et al. (1998). The ITS region was amplified with primers ITS1F and ITS4 (Gardes and Bruns, 1993; White et al., 1990) in PCR reactions containing 150 ng of DNA template, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer, and 1 U Taq polymerase (VWR International, Darmstadt, Germany). Temperature cycles consisted of a denaturation step at 94 °C for 4 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final elongation step of 72 °C for 5 min. For *β-tub* the primers β-tubulin_F and β-tubulin_R (Park et al., 2012) and the PCR protocol published by Thon and Royse (1999) were used. PCR products were checked by gel electrophoresis and sequenced using the same primers at GATC Biotech (Konstanz, Germany). The sequences obtained in this study are deposited in GenBank under accession numbers KU310900–KU310903 (Table S1).

Two independent phylogenetic reconstructions were performed for the ITS region and the *β-tub*. Appropriate reference sequences

were retrieved from GenBank, representing the *Ganoderma* species with ITS sequences closest to M9720 and M9724 in a preliminary BLAST analysis (Altschul et al., 1990) and *Coriolopsis caperata* as outgroup (Table S1). Sequences for each locus were first aligned using MAFFT v7.123b (Katoh and Standley, 2013) with the G-INS-i parameters, and then ambiguous regions were removed from alignments with Gblocks v0.91b (Castresana, 2000), allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions. Maximum Likelihood (ML) analyses were performed with RAxML v8.0.0 (Stamatakis, 2006), using a GTRGAMMA model of nucleotide substitution and 1000 bootstrap replicates. Posterior probabilities (PP) were estimated with MrBayes v3.2.2 (Huelsenbeck and Ronquist, 2001) using the same model. Two parallel searches were run for 10 M generations, with starts from random trees and sampling every 100th generation. The convergence of the runs was checked using TRACER v1.6 (Drummond and Rambaut, 2007). The first 30% of the resulting trees were discarded as burn-in, and PP were calculated from the remaining sampled trees.

4.4. Extraction and liquid chromatography/mass spectrometry

The extraction from dried, ground basidiocarps of strains M9720 and M9724 was performed with ethanol using a Soxhlet apparatus for 4 h. Removal of solvent in vacuum afforded ~400 mg of a brown extract (yield: 7.7%) from M9724, and ~300 mg of extract (yield: 6.0%) from M9720. The identification of derivatives was performed by ultra-performance liquid chromatography–electrospray ionization mass spectrometry (UPLC–ESIMS) in an Ultimate 3000 system (Dionex, Idstein, Germany) by using a RP C18 BEH Acquity UPLC column (50 mm × 2.1 mm × 1.7 µm, Waters, USA), coupled with a diode array detector and an Impact II Q-ToF (Bruker, Bremen, Germany) using a linear gradient from 5% to 95% ACN (0.1% formic acid) over 22 min with a flow rate of 0.6 ml min^{-1}. MS data was obtained both in positive and negative mode, with signals being much stronger in negative mode due to the presence of the carboxylic acid moiety. Therefore, data in negative mode were used for further analysis.

Acknowledgements

We express our gratitude to Brunhilde and Erwin Noll (Pilzfarm Noll) for supplying the dry basidiocarps, the ground basidiocarp powder of the strains M9720 and M9724, and answers to numerous questions. Jürgen Kynast (Pilzzucht Hesse) is kindly thanked for supplying information on the spawn substrate composition, preparation, inoculation, and incubation. We thank Magda Verfaillie (Mycelia bvba, Belgium) for providing information on M9720 and M9724. We also thank Peter Grün for technical assistance. This study was supported by LOEWE (Landes-Offensive zur Entwicklung wissenschaftlich-ökonomischer Exzellenz) of the state of Hesse and was conducted within the framework of the Cluster for Integrative Fungal Research (IPF).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2016.03.012.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Anonymous, 1955. Shen Nong materia medica, 102-200 A.D. (E. Han). Reprinted. People's Hygiene Press, Beijing.

Baby, S., Johnson, A.J., Govindan, B., 2015. Secondary metabolites from *Ganoderma*. Phytochemistry 114, 66–101.

Boh, B., Berovic, M., Zhang, J., Zhi-Bin, L., 2007. *Ganoderma lucidum* and its pharmaceutically active compounds. Biotechnol. Annu. Rev. 13, 265–301.

Brakhage, A.A., Schroeckh, V., 2011. Fungal secondary metabolites – strategies to activate silent gene clusters. Fungal Genet. Biol., Special Issue: Fungal Secondary Metabolism 48, 15–22.

Cao, Y., Wu, S.H., Dai, Y.C., 2012. Species clarification of the prize medicinal *Ganoderma* mushroom "Lingzhi". Fungal Divers. 56, 49–62.

Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.

Chen, S., Xu, J., Liu, C., Zhu, Y., Nelson, D.R., Zhou, S., Li, C., Wang, L., Guo, X., Sun, Y., Luo, H., Li, Y., Song, J., Henrissat, B., Levasseur, A., Qian, J., Li, J., Luo, X., Shi, L., He, L., Xiang, L., Xu, X., Niu, Y., Li, Q., Han, M.V., Yan, H., Zhang, J., Chen, H., Lv, A., Wang, Z., Liu, M., Schwartz, D.C., Sun, C., 2012. Genome sequence of the model medicinal mushroom *Ganoderma lucidum*. Nat. Commun. 3, 913.

Cheng, C.R., Yue, Q.X., Wu, Z.Y., Song, X.Y., Tao, S.J., Wu, X.H., Xu, P.P., Liu, X., Guan, S. H., Guo, D.A., 2010. Cytotoxic triterpenoids from *Ganoderma lucidum*. Phytochemistry 71, 1579–1585.

Cheng, C., Yang, M., Yu, K., Guan, S., Tao, S., Millar, A., Pang, X., Guo, D., 2012. Identification of metabolites of ganoderic acid D by ultraperformance liquid chromatography/quadrupole time-of-flight mass spectrometry. Drug Metab. Dispos. 40, 2307–2314.

Cheng, C.R., Yang, M., Yu, K., Guan, S.H., Wu, X.H., Wu, W.Y., Sun, Y., Li, C., Ding, J., Guo, D.A., 2013. Metabolite identification of crude extract from *Ganoderma lucidum* in rats using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry. J. Chromatogr. B 941, 90–99.

Cole, R.J., Jarvis, B.B., Schweikert, M.A., 2003. Handbook of Secondary Fungal Metabolites. Academic Press, San Diego.

Curtis, W., 1781. Flora Londinensis: or plates and descriptions of such plants as grow wild in the environs of London. London: printed by the author, 530 pp.

Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.

Ferreira, I.C.F.R., Heleno, S.A., Reis, F.S., Stoijkovic, D., Queiroz, M.J.R.P., Vasconcelos, M.H., Sokovic, M., 2015. Chemical features of *Ganoderma* polysaccharides with antioxidant, antitumor and antimicrobial activities. Phytochemistry 114, 38–55.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118.

Hawksworth, D., 2013. What is the Chinese Lingzhi *Ganoderma*? IMA Fungus 4 (47).

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Katoh, K., Standley, D.M., 2013. MAFFT: multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780.

Keypour, S., Rafati, H., Riahi, R., Mirzajani, F., Moradali, M.F., 2010. Qualitative analysis of ganoderic acids in *Ganoderma lucidum* from Iran and China by RP-HPLC and electrospray ionisation-mass spectrometry (ESI-MS). Food Chem. 119, 1704–1708.

Kirk, P.M., Cannon, P.F., Minter, D.W., Stalpers, J.A., 2008. Ainsworth & Bisby's dictionary of the fungi. CABI Europe, UK, Wallingford, Oxon.

Kubota, T., Asaka, Y., Miura, I., Mori, H., 1985. Structures of ganoderic acid A and B, two new lanostane type bitter triterpenes from *Ganoderma lucidum* (Fr.) Karst. Helv. Chim. Acta 65, 611–619.

Li, J.Q., Zhan, J.H., Chen, H.M., Chen, X.D., Lan, J., Liu, C., 2013. Complete mitochondrial genome of the medicinal mushroom *Ganoderma lucidum*. PLoS ONE 8, e72038.

Mims, C.W., Seabury, F., 1989. Ultrastructure of tube formation and basidiospore development in *Ganoderma lucidum*. Mycologia 81, 754–764.

Moncalvo, J.M., Wang, H.F., Hseu, R.S., 1995a. Gene phylogeny of the *Ganoderma lucidum* complex based on ribosomal DNA sequences: comparison with traditional taxonomic characters. Mycol. Res. 99, 1489–1499.

Moncalvo, J.M., Wang, H.F., Wang, H.H., Hseu, R.S., 1995b. The use of ribosomal DNA sequence data for species identification and phylogeny in the Ganodermataceae, in: Buchanan, P.K., Hseu, R.S., Moncalvo, J.M. (Eds.), Ganoderma: Systematics, Phytopathology and Pharmacology, Proceedings of Contributed Symposium 59A, B. 5th International Mycological Congress, Vancouver, 14–21 August 1994, pp. 31–41.

Nishitoba, T., Sato, H., Sakamura, S., 1985. New terpenoids from *Ganoderma lucidum* and their bitterness. Agric. Biol. Chem. 49, 1547–1549.

O'Donnell, K., Cigelnik, E., Nirenberg, H.I., 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90, 465–493.

Park, Y.-J., Kwon, O.-C., Son, E.-S., Yoon, D.-E., Han, W., Yoo, Y.-B., Lee, C.-S., 2012. Taxonomy of *Ganoderma lucidum* from Korea based on rDNA and partial β-tubulin gene sequence analysis. Mycobiology 40, 71–75.

Pegler, D.N., 2002. Useful fungi of the world: the Ling-zhi – the mushroom of immortality. Mycologist 16, 100–101.

Core Team, R., 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Richter, C., Wittstein, K., Kirk, P.M., Stadler, M., 2015. An assessment of the taxonomy and chemotaxonomy of *Ganoderma*. Fungal Divers. 71, 1–15.

Ryvarden, L., 2000. Studies in neotropical polypores 2: a preliminary key to neotropical species of *Ganoderma* with a laccate pileus. Mycologia 92, 180–191.

Shiao, M.S., 2003. Natural products of the medicinal fungus *Ganoderma lucidum*: occurrence, biological activities, and pharmacological functions. Chem. Rec. 3, 172–180.

Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.

Thon, M.R., Royse, D.J., 1999. Partial ß-tubulin gene sequences for evolutionary studies in the Basidiomycotina. Mycologia 91, 468–474.

Wachtel-Galor, S., Tomlinson, B., Benzie, F.F., 2004. *Ganoderma lucidum* ('Lingzhi'), a Chinese medicinal mushroom: biomarker responses in a controlled human supplementation study. Br. J. Nutr. 91, 263–269.

Wang, D.M., Wu, S.H., Su, C.H., Peng, J.T., Shih, Y.H., Chen, L.C., 2009. *Ganoderma multipileum*, the correct name for '*G. lucidum*' in tropical Asia. Bot. Stud. 50, 451–458.

Wang, X.C., Xi, R.J., Li, Y., Wang, D.M., Yao, Y.J., 2012. The species identity of the widely cultivated *Ganoderma*, '*G. lucidum*' (Ling-zhi), in China. PLoS ONE 7, e40857.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Orlando, Florida, pp. 315–322.

Yang, Z.L., Feng, B., 2013. What is the Chinese "Lingzhi"? – a taxonomic mini-review. Mycology. http://dx.doi.org/10.1080/21501203.2013.774299.

Yang, M., Wang, X., Guan, S., Xia, J., Sun, J., Guo, H., Guo, D.A., 2007. Analysis of triterpenoids in *Ganoderma lucidum* using liquid chromatography coupled with electrospray ionization mass spectrometry. J. Am. Soc. Mass Spectrom. 18, 927–939.

Yao, Y.J., Wang, X.C., Wang, B., 2013. Epitypification of *Ganoderma sichuanense* J.D. Zhao & X.Q. Zhang (Ganodermataceae). Taxon 62, 1025–1031.

Zhao, J., Zhang, X.Q., Li, S.P., Yang, F.Q., Wang, Y.T., Ye, W.C., 2006. Quality evaluation of *Ganoderma* through simultaneous determination of nine triterpenes and sterols using pressurized liquid extraction and high performance liquid chromatography. J. Sep. Sci. 29, 2609–2615.

Zhou, L.W., Cao, Y., Wu, S.H., Vlasák, J., Li, D.W., Li, M.J., Dai, Y.C., 2015. Global diversity of the *Ganoderma lucidum* complex (Ganodermataceae, Polyporales) inferred from morphology and multilocus phylogeny. Phytochemistry 114, 7–15.