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IS THE WIDELY USED MEDICINAL FUNGUS THE *GANODERMA LUCIDUM* (FR.) KARST. *SENSU STRICTO*?

(A SHORT REVIEW)

GYÖNGYI SZEDLAY

Department of Plant Anatomy, Eötvös Loránd University, Pázmány Péter sétány 1/C H–1117 Budapest, Hungary

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The identification of *Ganoderma* species is usually based on classical morphological criteria. The objectives of this review were to collect available information on *Ganoderma lucidum* and to utilize them in exact identification of *Ganoderma lucidum* (Fr.) Karst. *sensu stricto*. A lot of taxonomical confusion has always been associated with *G. lucidum* and allied species. Species circumscription, phylogenetic relationships, host range and distribution of species of the *G. lucidum* complex are unclear even among the few taxa living in temperate climate. Several methods have been proposed to identify the species as examination of cultural characteristics, isozymes, secondary metabolites, DNA sequences and interfertility. Although *G. lucidum sensu stricto* has been reported world-wide accumulated evidence supported the suggestion that it seems restricted to Europe. The strains used in the medicine are usually collected in Asia. There is little likelihood that any one belongs to the *G. lucidum sensu stricto*. The strains labelled as *G. lucidum* in the medicinal and pharmacological literature encompass a broad range of species which produce different medicinally active compounds and have significantly different pharmacological effects.

Keywords: *Ganoderma lucidum*, taxonomy, lanostanoids, chemotaxonomy

Introduction

"*Ganoderma* genus is currently in a state of taxonomical chaos." Ryvarden [1].

In Chinese folklore *Ganoderma* has been regarded as a panacea for many types of diseases. The screening efforts for medicinally effective agents proved that

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Ganoderma extracts enhance the immune system without considerable side effects due to its polysaccharides. Many biological activities associate with oxygenated terpenoids characteristic to the genus called lanostanoids [2, 3].

Since 1996 plenty of papers have been published concerning the laccate *Ganoderma* as a medicinal fungus in different fields (new effective metabolites, medicinal effects, cultivation for medicinal use, etc.) by the well-known international periodicals. 143 of them identified the species examined as *G. lucidum* (Fr.) Karst. Many of them do not contain any information about the method of the identification of the species, although the authors of few papers identified the fungus and deposited the strain or the fruit body in a scientific institute. Occasionally fruit bodies bought in the local markets were used. The method of species identification was not mentioned in the papers. The use of strains originating from an authentic strain collection was reported only in three cases. The identity of specimens is often uncertain in these medicinal studies.

The exact identification of the specimens used for any medicinal purposes would be essential. In the traditional Chinese books *Ganoderma* was classified into six species according to the colour of the fruit body: Sekishi is red, Shishi violet-like, Kokushi black, Oushi yellow, Hakushi white and Seishi blue. These have been assigned to have different pharmaceutical effects which was proved to base on different triterpenoid pattern. The HPLC analysis of the acidic fraction resulted similar triterpenoid pattern of Sekishi and Shishi. The main lanostanoid component depended on the strain. Kokushi had a unique triterpenoid pattern. The exact influence of taxonomical differences on triterpenoid pattern has been examined but not clarified yet as it is explained later. Many experiments confirmed that the quality and the quantity of the lanostanoids depend on the specimens examined [4, 5, 6]. The method of identification should be unambiguous in fruit bodies and also in cultures, fast, easy to evaluate and simple to make the exact identification possible for the pharmaceutical companies and chemical laboratories.

Possibilities in the identification of *Ganoderma* species

Morphology and anatomy of the fruit bodies and the cultures

The exact identification of the laccate *Ganoderma* specimens is a difficult task even for the experts of this field. The diversity within the genus *Ganoderma* is high. About 250 species have been described sometimes based on only a single collected specimen [1]. *Ganoderma* species are white-rot polypore fungi. The *G. lucidum* complex is composed of species with annual basidiocarps varying in colour from yellow, orange-red to black with several brownish gradations. The laccate resin is produced by the outer layer of the pileus composed of pilocystidia. Basidiocarps are stipitate or sessile. The context is uniformly coloured or duplex, ranging from nearly white to brown. The pore surface is light-coloured. Basidiospores are double-walled with interwall pillars.

Taxonomical characteristics used widely within the species group are macromorphology of the fruit body, spore characteristics, geographical distribution and host specificity. The species identification within the *G. lucidum* complex based on these characteristics has resulted confusion in identification because of the similarities among the species and diversity within the species.

Some of the characteristics of the fruit body were found to be pleomorphic. The context colour became darker with southern latitudes and lower altitudes. These observations suggested the influence of temperature on morphological characters [8]. Dikaryotic cultures from sessile fruit bodies of *G. lucidum* produced stipitate fruit bodies [9].

Pilocystidia are the hyphal elements that compose the outer layer of the pileus. Adaskaveg and Gilbertson [10] examined five North-American species. Species of the *G. lucidum* complex have pilocystidia with inflated ends. These cells are all embedded in laccate resin and ranged from long and cylindrical to short and clavate to capitate. Pilocystidia were morphologically different in the species studied. These are valuable characters in separating species but the method is unfortunately time-consuming, and require scanning electron microscopy which is not widely used in pharmaceutical examinations.

The spore characteristics are also often uncertain. The spore size depends on the latitude and altitude as Steyaert [8] found in *G. tornatum* (Pers.) Bres. The range of the spore index and the spore width had considerable overlap among the similar species. Microscopically the outer wall surface roughness and interwall-pillar sizes were different. The natural variation within the population made indistinguishable the species by the statistical analysis of these characters [9, 11].

Host specificity of *Ganoderma* species is not completely understood. *In vitro* wood decay studies with isolates of *G. lucidum* and *G. tsugae* Murr. have indicated that both species are capable of decaying wood of conifers or hardwoods [12], although *G. lucidum in vivo* lives on hardwood and *G. tsugae* on conifers.

Nobles [13, 14] suggested to use the less variable cultural characteristics in the description of the fungal species. Differences were indicated in the cultural characteristics of *G. lucidum* and *G. tsugae*. The characters most useful in distinguishing between *Ganoderma* cultures are chlamydospore production, growth rate and thermophily [9]. There is a remarkable correlation between these characters: fast-growing cultures are thermophilic and produce numerous ovoid chlamydospores, while slow-growing cultures are not thermophilic and do not produce chlamydospores. The use of these characteristics for the indentification of the species is only possible together with other examinations because distant species may share same values and characters [15]. Morphological characters and cultural features alone appear insufficient to distinguish the different species of *Ganoderma*. New parameters are required to allow the detection of interspecific and intraspecific variability.

Isozymes

The systematic revision of the genus using isozyme electrophoresis of enzyme-systems has been undertaken. The problem of the method was the difficulty of recognizing discontinuities among the species [16, 17]. Pectic enzymes using PAGE showed promise in identifying laccate species [18].

Secondary metabolites

Many secondary metabolites have been used as marker compounds for the characterization and classification of taxonomically related fungi. Those metabolites are suitable which are not greatly subjected to seasonal variations or changes of culture conditions. These are the lanostanoids in *Ganoderma*. The triterpenoid patterns are potentially applicable in the chemotaxonomy of the genus *Ganoderma*. Chyr and Shiao [19] used 25 triterpenoids to characterize the methane extract of the different strains by HPLC. The triterpenoid patterns remained unchanged at least until 50 days. The triterpenoid pattern and the morphological characterization are generally in good agreement.

Chen et al. [6] compared the ganoderic acid A, B, C and D content in ethane extracts of *G. lucidum* and *G. tsugae* strains by HPLC. The different strains of *G. lucidum* displayed different triterpenoid patterns while *G. tsugae* strains displayed similar spectra. The patterns of the different species were definitely different.

The fastest and simplest method was used by Su et al. [20]. Ethane extracts of 64 different strains were examined. Two external standards of ganoderic acids B and C2 were used. The samples were divided into groups based on the following criteria: the existence of chromatographic peaks for ganoderic acids B and C2, the peak number before ganoderic acid B and after ganoderic acid C2, the peak number between ganoderic acid B and C2, the total peak number in a chromatographic period when there were no peaks of ganoderic acids B and C2. This results in a well definite grouping of strains. The most interesting result of this work is that the TLC chromatograms of these extracts showed a similar grouping pattern as by HPLC. The TLC is used as standard method for examinations of drugs in the European pharmacopoeia and almost in each national pharmacopoeia. The advantage of standardization of this method in the official identification in pharmacology would be obvious. Although TLC and HPLC analyses of the triterpenoid contents of *Ganoderma* seem to be a simple and easy method to differentiate the species of the genus, there are also serious difficulties. The terpenoid patterns obtained by HPLC analysis of the acidic fractions of the pilei of different growth stages were very similar to each other. The main components were ganoderic acid A, B and H. Nevertheless, ganoderic acid R, S and T was found to be dominant in the underground parts and cultured micelium. This suggests that the expression of the metabolic ability correlates strictly with fungal differentiation [21]. The quantity and the quality of the triterpenoids of the entire fruit body change during fruit body formation [4]. Nomenclature relies on basidiocarps of type specimens, of which, usually, no culture was derived. This method is very useful if we compare culture collections cultivated in identical conditions but not suitable for examinations of type specimens, culture collections and fruit bodies of different ages.

DNA sequences

The use of molecular data, where other characteristics are dubious, has been proposed by many authors. Moncalvo et al. [15] inferred natural relationships by comparison of the internal transcribed spacer regions (ITS1 and ITS2) and divergent region D2 of the large ribosomal subunit gene (LSU-D2) nucleotide sequence. He found six monophyletic lineages, but the basal relationships were not resolved among them. The ITS dataset provided phylogenetic information at lower taxonomical levels while the LSU dataset was more useful at higher levels. On lower taxonomic levels monophyly of 11 groups was strongly supported by the bootstrap analysis. Nucleotide sequence data showed that ITS nucleotide sequence variation among *Ganoderma* species provides taxonomic resolution around the species level [5]. This method is easy for use due to the international sequence databases, unambiguous, fast and simple, but

the identification of monophyletic groups does not always mean the identification of biological species.

Interfertility tests

Nobles [13] suggested to use interfertility tests to determine the identity of the species. Up to now, interfertility tests with homokaryons have been carried out only with few *Ganoderma* species. In the case of *Ganoderma* species homokaryons are difficult to obtain because the low percentage or total lack of basidiospore germination. The species of the *G. lucidum* complex examined in this respect so far have heterothallic, tetrapolar mating system. None of the North American *G. tsugae* homokaryons were interfertile with the North American *G. lucidum* homokaryons [9]. Sheu's [15] experiments had the same results in connection with the Asian strains identified as *G. lucidum* and *G. tsugae*. The two taxa are intersterile. Homokaryons of the European *G. resinaceum* Boud. isolate were completely interfertile with homokaryons of the North American *G. lucidum* [9]. This is inconsistent with the result of ITS sequencing which excluded the conspecificity of the two strains examined in the mating studies. This result raised the issue that *in vitro* intercompatible strains are not necessarily intercompatible *in vivo* and belong to the same species [7].

Is the widely used medicinal fungi the *Ganoderma lucidum sensu stricto*?

To answer this question we had to examine the literature about *G. lucidum* in the respects listed above. *G. lucidum* was described from Europe. As a consequence we regarded European *G. lucidum* strains as *G. lucidum sensu stricto*. The morphology of the fruit body is uniform among the fungi from different locations.

The European *G. lucidum* strains have clavate and unbranched pilocystidia. The apical part is smooth and spherical. No other cells take part in forming the structure of the surface layer [22]. The surface tissue of basidiocarps of *G. lucidum* isolated in North America is composed of a dense palisade of pilocystidia, however, branching non-swollen hyphae are frequently intermixed. Pilocystidia are amyloid, thick-walled, and clavate with abruptly tapering shafts that are occassionally branched. Pilocystidial apices are mostly smooth, although some have several knob-like projections [10].

Those strains, growing in different regions but assigned to *G. lucidum* show quite a different distribution pattern for their triterpenoid content [20].

The ITS gene phylogeny shows that isolates identified as *G. lucidum* do not cluster together [7]. The *G. lucidum* strains originated from the same continent formed clades. The South American *G. lucidum* isolates have identical ITS sequences and

show considerable differences compared with those of *G. lucidum* from Europe, North America and Philippines [23].

Cultural features (Table I), phylogenetic relationships and triterpenoid patterns support the distinction between European, Asian, South and North American *G. lucidum* strains. Results collected up to now indicate that *G. lucidum sensu stricto* is geographically restricted to Europe and presumably absent from America and from Eastern Asia (we have no results about North and West Asian *Ganoderma* strains). The species might be too young to have spread worldwide [15]. The morphological features traditionally used for *Ganoderma* systematics show convergence and paralellism. Research in pharmacology of *Ganoderma* is largely concentrated in Asia. There is little likelihood that the strains used for these purposes belong to the *G. lucidum sensu stricto*. According to the above mentioned differences the strains labelled as *G. lucidum* in the medicinal and pharmacological literature encompass a broad range of species which produce different medicinally active compounds and have significantly different pharmacological effects.

Table I

The growth characteristics of cultures on MEA identified as Ganoderma lucidum

Species	Origin	Optimal temperature range/ ^{o}C	Growth interval/ ^{o}C	Growth rate mm/day	Chlamydo-spores in culture
G. lucidum [9]	North America	30–34	<10–42	7–11	present
G. lucidum [22]	Europe	22–26	10–30	11	absent
G. lucidum [15]	Philippines, Taiwan, India	28–32	still growing at 38 ^{o}C	>7	present
G. lucidum	cultivated in China	no data	no data	no data	absent
G. lucidum [23]	South America	no data	no data	no data	absent

Which method is reliable in the identification of the species?

Since morphology in *G. lucidum* complex was found to be too variable to distinguish the species, a complex examination is suggested. The most suitable features are the ITS sequences, the cultural characteristics mentioned in Table I and the triterpenoid patterns.

As a consequence, numerous collections among the strains used have to be renamed. The use of exact nomenclature will be possible if the type specimens are studied at the molecular level besides comparative morphological studies of basidiocarp characteristics. To resolve the contradiction between morphological and phylogenetical species mating tests have to be conducted. This is in progress in our laboratory, and the first results will be published in the near future.

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