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Delimitation of some neotropical laccate *Ganoderma* (Ganodermataceae): molecular phylogeny and morphology

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Abstract: *Ganoderma* includes species of great economic and ecological importance, but taxonomists judge the current nomenclatural situation as chaotic and poorly studied in the neotropics. From this perspective, phylogenetic analyses inferred from ribosomal DNA sequences have aided the clarification of the genus status. In this study, 14 specimens of *Ganoderma* and two of *Tomophagus* collected in Brazil were used for DNA extraction, amplification and sequencing of the ITS and LSU regions (rDNA). The phylogenetic delimitation of six neotropical taxa (*G. chalceum, G. multiplicatum, G. orbiforme, G. parvulum, G. aff. oerstedtii* and *Tomophagus colossus*) was determined based on these Brazilian specimens and found to be distinct from the laccate *Ganoderma* from Asia, North America and from some specimens from Argentina. Phylogenetic reconstructions confirmed that the laccate *Ganoderma* is distinct from *Tomophagus*, although they belong to the same group. The use of taxonomic synonyms *Ganoderma subamboinense* for *G. multiplicatum*, *G. boninense* for *G. orbiforme* and *G. chalceum* for *G. cupreum* was not confirmed. However, *Ganoderma parvulum* was confirmed as the correct name for specimens called *G. stipitatum*. Furthermore, the name *G. lucidum* should be used only for European species. The use of valid published names is proposed according to the specimen geographical distribution, their morphological characteristics and rDNA analysis. Rev. Biol. Trop. 62 (3): 1197-1208. Epub 2014 September 01.

Key words: Agaricomycetes, phylogenetic taxonomy, rDNA sequences, species delimitation, neotropics.

*Ganoderma* P. Karst. (Ganodermataceae, Agaricomycetes) is one of the largest genera of Polyporales and was described by Karsten (1881) based on *Polyporus lucidus* (Curtis) Fr. from Europe, a species with a laccate (shiny varnished looking) surface. The genus is characterized by double-walled basidiospores with truncate apex and ornamented endospore (Moncalvo & Ryvarden, 1997). The genus includes 80 species of wide geographic distribution with several tropical species and others restricted to temperate areas (Ryvarden, 2000; Kirk, Cannon, Minter, & Stalpers, 2008). Ryvarden (2004) reported the presence of 20 species in the neotropics, although Torres-Torres, Guzmán-Dávalos & Gugliotta (2012) and Gugliotta, Abrahão & Gibertoni (2013) listed 18 and 28 species, respectively, only in Brazil.

Being well known as decomposers and pathogens in tropical forests (Zakaria, Ali, Salleh, & Zakaria, 2009), species of this genus, mainly of the *G. lucidum* complex, produce bioactive compounds widely studied for preventing and relieving human diseases, such as several types of tumors and cancers, gastric ulcers, diabetes mellitus, hypertension and viral infections (Zhou et al., 2007). Besides, some medicinal effects were reported in manuscripts of the Chinese civilization more than 2000 years ago (Hong & Jung, 2004; Seo & Kirk, 2000).

Although of significant ecological and biotechnological importance, the taxonomy
of laccate Ganoderma has been questioned in recent years and poorly studied in the neotropics. Currently, taxonomists consider the nomenclatural situation as chaotic and suggest the necessity for a global revision due to the existence of multiple names for single species (Ryvarden, 1991; Ryvarden, 2004; Moncalvo & Ryvarden, 1997; Postnova & Skolotneva, 2010).

Usually, different morphological characteristics of the basidiospores (dimensions) and basidiomata (thickness of the cuticle and presence or absence of resinaceous deposits in the context) are widely used in an attempt to identify laccate species of Ganoderma (Seo & Kirk, 2000; Ryvarden, 2004; Torres-Torres & Guzmán-Dávalos, 2012; Torres-Torres, Guzmán-Dávalos, & Gugliotta, 2012). However, the use of molecular data, especially for phylogenetic studies based on ribosomal DNA sequences, combined with morphological studies has helped many authors to clarify the status of the genus (Moncalvo, Wang, & Hseu, 1995a; Moncalvo, Wang, & Hseu, 1995b; Gottlieb, Ferrer, & Wright, 2000; Smith & Sivathamparam, 2000; Hong & Jung, 2004; Kaliyaperumal & Kalaichelvan, 2008; Cao, Wu, & Dai, 2012; Yang & Feng, 2013).

In Brazil, there are only a few studies regarding Ganoderma (Torrend, 1920; Loguerocio-Leite, Groposo, & Halmenschlager, 2005; Torres-Torres et al., 2012) and none of these include a phylogenetic analysis. Thus, the aim of the present study was to determine phylogenetic relationships of the laccate Ganoderma based on sequence variation of the Internal Transcribed Spacer (ITS) and Large subunit (LSU) rDNA and to delimit the species’ occurrence within Brazilian territory.

MATERIAL AND METHODS

Morphological analysis: Thirty-one laccate specimens of Ganoderma and four of Tomophagus colossus (Fr.) Murrill were used in this study (Table 1). To observe the characteristics of the basidiospores, the hyphal system and the cuticle hyphae, free-hand thin sections of dried material were mounted in 5% KOH to ensure rehydration. Melzer’s reagent was used to test the amyloid reaction of the cuticle hyphae. Our data were then compared to the available literature (Bresadola, 1911; Gottlieb & Wright, 1999; Núñez & Ryvarden, 2000; Ryvarden, 1989; Ryvarden, 2004; Welti & Courtecuisse, 2010; Tham et al., 2012, Torres-Torres et al., 2012) and discussed in the text. All studied specimens were deposited in the herbarium Pe. Camille Torrend (URM), Department of Mycology, Universidade Federal de Pernambuco, Brazil.

Genomic DNA extraction, polymerase chain reaction and sequencing: DNA was extracted using fragments of basidiomata (30-50mg) ground with a pestle in a porcelain mortar containing liquid nitrogen. The resulting powder was transferred to a tube containing 700μL of extraction buffer [CTAB 2%, 100mM Tris-HCl pH8, 1.4M NaCl, 20mM EDTA, 1% PVP (Rogers & Bendich, 1985)] and incubated at 65°C for 30-40 min. DNA was purified with 700μL of chloroform-isoamyl alcohol (24:1), gently precipitated in 600μL of isopropanol and washed with 1mL of ethanol. Finally, the pellet was suspended in 50μL of ultrapure water (Góes-Neto, Loguercio-Leite, & Guerrero, 2005). The reaction mix and parameters for PCR amplification of the full ITS regions was according to Smith & Sivathamparam (2000) using the primers ITS1 and ITS4 (White, Bruns, Lee, & Taylor, 1990). For LSU rDNA region, the amplification was performed with parameters and reagent concentrations following Góes-Neto, Loguercio-Leite & Guerrero (2005) using the primer pair LR0R and reverse LR7 (Moncalvo, Lutzoni, Rehner, Johnson, & Vilgalys, 2000). Negative controls containing all components of the reaction mix, except DNA, were used in each procedure to detect possible contamination. The amplification products of the sixteen laccate specimens (Table 1) were purified using the PureLink PCR Purification Kit (Invitrogen) and the purified products were sequenced at the Human Genome Research Center of the Universidade
de São Paulo (USP, Brazil) in an ABI-310 Capillary Sequencer (PerkinElmer, Wellesley Massachusetts, USA). Cycle sequencing was carried out with primers ITS1 and ITS4 for ITS region and LR0R and LR5 for LSU region (Moncalvo et al., 2000). All sequences were deposited in GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA).

**Phylogenetic analysis:** Sixteen ITS and LSU rDNA sequences (14 of *Ganoderma* and two of *T. colossus*) of laccate Ganodermataceae were compared with other sequences retrieved
from GenBank (Table 2). These sequences were aligned using ClustalX (Larkin, 2007), manually edited in BioEdit (Hall, 1999) and realigned to obtain the final alignment. Phylogenetic analyses and tree construction were performed separately for each locus. Neighbor joining (NJ) distances, maximum parsimony (MP) and maximum likelihood (ML) analyses were carried out using PAUP* version 4.0b10 (Swofford, 2002) and the support was evaluated using 1 000 bootstrap replicates. The NJ and ML analysis were based on HKY+G (ITS) and TIM+I (LSU) obtained from ModelTest 3.7 (Posada & Crandall, 1998), which computed the most likely patterns of phylogenetic evolution. Sequences from Amauroderma rude var. intermedium J. S. Furtado were used as outgroup for phylogenetic reconstruction based on ITS sequences and two specimens of T. colossus were used as outgroup for the LSU analysis.

RESULTS

The ITS1-5.8S-ITS2 regions sequenced in this study varied in length from 548 to 571 nucleotides. The size of the ITS1 region did not differ markedly among the studied specimens, ranging from 199 to 205 nucleotides. This small variation was also observed for the ITS2 region and ranged between 192 and 201 nucleotides. The final alignment (ITS1 + ITS2) included 419 sites, with 266 constant sites (63%) and 153 variable (36%), of which 120 (28%) were parsimony informative.

The size of LSU sequences ranged from 1320 to 1322 nucleotides and aligned at 1323 positions. Of these, 1278 characters were constant, 21 characters were variable but parsimony uninformative, and 24 characters were parsimony informative. Although this study provided new sequences within the ITS and LSU regions, it is as yet impossible to perform multigene analysis due to the lack of other neotropical gene sequences from the species analyzed here.

The phylogenetic reconstruction performed with NJ, MP and ML analyses for ITS sequences showed basically the same topology and few differences in bootstrap values (Fig. 1). The same was observed for LSU analysis (Fig. 2). These results confirm that laccate Ganoderma is a monophyletic group although with low statistical support based on ITS analysis (NJ 70%; MP < 50% and ML 60%) and with high bootstrap values based on LSU analysis (NJ, MP and ML 100%). In the phylogenetic reconstruction based on ITS regions, seven clades were delimited (A, B, C, D, E, F and G). The Brazilian specimens of the six taxa studied (G. chalceum, G. multiplicatum, G. orbiforme, G. parvulum, G. aff. oerstedii and T. colossus) were recovered in clades A, B, D and G, discussed below.

DISCUSSION

The clade A formed a monophyletic lineage distinct from the laccate Ganoderma with strong statistical support (NJ, MP and ML = 100%) and included two species, T. colossus and T. cattienensis. Tomophagus colossus from Brazil grouped with representatives of the Asian species (NJ 98%, MP 99% and ML 90%), confirming that they are of the same species, but distinct from T. cattienensis. The genus Tomophagus was established first by Murrill (1905) and in the following decades was contested, being considered a confusing group. Later, molecular phylogenetic studies confirmed the genus as group well established in Ganodermataceae (Moncalvo, Wang, Wang, & Hseu, 1995c; Hong & Jung, 2004; Tham et al., 2012).

However, recent papers still mention Tomophagus as belonging to Ganoderma (Welti & Courtecuisse, 2010; Cao et al., 2012). Tomophagus currently has two species, T. colossus (= G. colossus) and T. cattienensis, both sharing the laccate (shiny) pilear surface, pale context with slightly dextrinoid skeletal hyphae, large basidiospores, and the striking chlamydospores, providing a unique combination of characters (Ryvarden, 2000; Ryvarden, 2004; Tham et al., 2012). Tomophagus colossus differs morphologically from T. cattienensis by having yellowish pilear surface while T. cattienensis has red to light brown pilear surface.
### TABLE 2
Origin and GenBank accessions of the strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Geographic Origin</th>
<th>Strain/specimen number</th>
<th>GenBank accession number</th>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>X78749&amp;X78770</td>
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<td>G. cupreum</td>
<td>Australia</td>
<td>DFP 3896</td>
<td>AJ627586 &amp; AJ627587</td>
</tr>
<tr>
<td>G. cupreum</td>
<td>Australia</td>
<td>DFP 4336</td>
<td>AJ627588 &amp; AJ627589</td>
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<td>AF170009&amp;AF170010</td>
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<td>G. lucidum</td>
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<td>Z37062 &amp; Z37085</td>
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<td>X78736&amp;X78757</td>
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<td>AF170011&amp;AF170012</td>
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<td>HCMC10 (TRTC 161190)</td>
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<td>CT99 (TRTC 161191)</td>
<td>JN184397</td>
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<td>CT119</td>
<td>JN184398</td>
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<td>X78777</td>
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<td>n.a</td>
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<td>Korea</td>
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<td>Korea</td>
<td>C-1 (Wild)</td>
<td>DQ208412</td>
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<td>G. lucidum</td>
<td>Korea</td>
<td>C-2 (Wild)</td>
<td>DQ208413</td>
</tr>
</tbody>
</table>

n.a= not available.
Fig. 1. Phylogenetic reconstruction of the laccate *Ganoderma* based on alignment of 419 nucleotides of the ITS region. Bootstrap values (%) were generated from neighbor joining method with distances of HKY+G, maximum parsimony and maximum likelihood (ML) analysis (1 000 bootstraps), respectively. Values above 50% are shown. One specimen of *Amauroderma rude* var. *intermedium* was used as outgroup. For maximum parsimony: Consistency Index (CI) = 0.6690 and Retention Index (RI) = 0.8898.
Furthermore, *T. cattienensis* has a context that turns pale brown upon drying, instead of remaining creamy white as *T. colossus*, and shows slightly larger basidiospores [17.5-21.5 x 11.5-14.5μm (Tham et al., 2012) versus 16-19 x 10.5-12.5μm in *T. colossus* from Brazil]. This size is consistent with Ryvarden’s (2004) descriptions.

Clade B was composed of specimens of *G. multiplicatum*, *G. multipileum*, *G. parvulum*, *G. tuberculosum* and *G. lingzhi*. Similar topology was also observed in the phylogenetic analysis based on LSU sequences for the available species (*G. multiplicatum*, “*G. lucidum*”, *G. parvulum*) (Fig. 2).

*Ganoderma multiplicatum* is a neotropical species described from Venezuela and is characterized by having amyloid, slightly tuberculate hyphal ends in the cuticle, and sub-globose to ellipsoid basidiospores (7.5-8.5 x 5-6μm). The Brazilian specimens studied agree with the descriptions of Gottlieb & Wright (1999), Ryvarden (2000, 2004) and Torres-Torres, Guzmán-Dávalos, & Gugliotta (2012). Ryvarden (2000) recognized *G. subamboinense* Henn. as a synonym of *G. multiplicatum*, originally described from Brazil. Although morphologically similar [context with two or more black, resinous layers, hyphal ends in the cuticle generally amyloid and basidiospores

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Fig. 2. Phylogenetic reconstruction of the laccate *Ganoderma* from Brazil based on alignment of the 1323 nucleotides of the LSU region. Bootstrap values (%) were generated from neighbor joining method with distances of TIM+1, maximum parsimony and maximum likelihood analysis (1000 bootstraps), respectively. Values above 50% are shown. Two specimen of *T. colossus* were used as outgroup. For maximum parsimony: Consistency Index (CI) = 0.7966 and Retention Index (RI) = 0.8519.
8-10 x 6-7μm (Gottlieb & Wright, 1999)], G. subamboinense Henn var. laevisporum Bazzalo & J.E. Wright ATCC 52419 (with a single sequence available for the species) were shown here to be distantly related (clade E), and thus should not be considered as synonyms.

Of the specimens here identified as G. parvulum by morphology and phylogenetic analysis, one was deposited in Herbarium URM as G. stipitatum (URM 80765) and another in the Culture Collection URM as G. resinaceum (URM 2948). For a long time, G. parvulum was considered synonym of G. stipitatum (Moncalvo & Ryvarden, 1997; Ryvarden, 2004). We agree that these are the same species, however, we follow the opinion of Torres-Torres et al. (2012) that the name G. parvulum Murrill 1902 should be used in preference to G. stipitatum (Murrill) Murrill 1908 (Fomes stipitatus Murrill 1903) as it was described earlier. Steyaert (1980) cited a wrong reference for the G. parvulum protologue and this was followed by Ryvarden (2004).

Ganoderma resinaceum URM 2948 is probably G. parvulum incorrectly identified. The basidiomata of G. resinaceum used for the original identification of the strain was not deposited in any Herbarium, preventing re-identification of the specimen. These two species are macromorphologically similar, and, microscopically, they both have smooth, weakly amyloid hyphal ends of the cuticle. However, G. resinaceum has larger basidiospores [9-11.5 x 5-7μm according to Ryvarden (2004) and 11.2-12.5 x 6.5-7.4μm according to Torres-Torres et al. (2012) versus 8-10 x 5-6μm in G. parvulum from Brazil] and no black, resinous layers in the pale brown context (Ryvarden, 2004; Torres-Torres et al., 2012). The basidiospores of the Brazilian specimens of G. parvulum were similar to the description in Ryvarden (2004, as G. stipitatum). Furthermore, G. resinaceum was described on the basis of a specimen from France and G. parvulum was originally described from Brazil.

Similarly, the Argentinean specimen described as G. tuberculosum (BAFC 33599) (Gottlieb & Wright, 1999) also corresponds to G. parvulum. Ganoderma tuberculorum is characterized by a black, thick resinose band in the context similar to G. parvulum, but has longer basidiospores [10-11 x 7.5-9μm according to Welti & Courtecuisse (2010) versus 8-10 x 5-6μm in G. parvulum from Brazil]. Besides, G. tuberculorum does not have amyloid cuticle elements unlike G. parvulum, whose elements are distinctly amyloid. Gottlieb & Wright (1999) commented that the specimen BAFC 33599 was preliminarily identified as G. resinaceum, but was later revised to G. tuberculorum. However, they observed cuticle hyphal ends distinctly amyloid characteristic of G. parvulum and different from what was observed in the type of G. tuberculorum deposited in the Herbarium of the New York Botanical Garden in the study by Welti & Courtecuisse (2010). Yet, Gottlieb & Wright (1999) noted basidiospores ovoid to ellipsoid, 10-12 x 6-9μm, in three Argentinian specimens, including BAFC 33599, similar to the observed in G. tuberculorum. It is likely that the size of basidiospores is not a taxonomic criterion relevant for separating these two species, but the presence or absence of amyloid reaction of the cuticle elements is.

The specimens of G. parvulum and G. multiplicatum proved to be distinct from the specimens previously listed as G. lucidum for Asia (G. multiplicatum and G. lingzhi in figure 1; G. lucidum in Fig. 2) and from specimens still regarded as G. lucidum in South America, North America and Europe (clades C, E and F, respectively, in Fig. 1). Due to the high phenotypic plasticity of the subgenus Ganoderma, several species have been mistaken for G. lucidum strictu sensu. Torres-Torres et al. (2012) commented that Brazilian specimens of G. multiplicatum and G. parvulum have been mistaken for G. lucidum.

Although reports of G. lucidum in the neotropics are still found in the literature (Torres-Torres et al., 2012; Vasco-Palacios & Franco-Molano, 2013), in Herbaria records (http://emuweb.fieldmuseum.org/botany/crfResultList.php, http://splink.cria.org.br) and in online databases (http://www.cybertruffle.
In clade D, the specimen of *G. chalceum* grouped with one Brazilian specimen of *G. aff. oerstedii* (URM83400) (NJ 92%, MP 97% and ML 100%, for ITS analysis), both belonging to the *G. resinaceum* complex. This clade can also be observed in the phylogenetic reconstruction based on LSU sequences, although with low statistical support (NJ, MP an ML < 50%). Basidiospores are smaller in *G. chalceum* [10-12 x 5-7μm versus 12-15 x 8-10μm in *G. aff. oerstedii* (Ryvarden, 2004)] and the black resinous layer in the context is absent in *G. aff. oerstedii* (present in *G. chalceum*). Thus, in this clade, the size of basidiospores and presence of resinous deposits in the context seem to be important characters for species delimitation. *Ganoderma aff. oerstedii* URM83400 was initially identified as *G. resinaceum*, but the use of *G. resinaceum* for South America specimens is not appropriate since two specimens of *G. resinaceum* (type locality: France) from Europe (clade E) are distinctly related. The material of *G. aff. oerstedii* (URM83400) is scarce and more collections are desirable in order to confirm this species.

Clade G is composed by specimens of *G. orbiforme* and *G. cupreum*. All specimens of *G. orbiforme* clustered with high statistical support (ITS analysis: NJ 99%, MP and ML 100%; LSU analysis: NJ 97%, MP 78% and ML 74%) and were clearly distinct from the other laccate species.

The Index Fungorum databases and Ryvarden (2004) recognize *G. boninense* as synonym of *G. orbiforme*. However, the Mycobank database considers *G. boninense* as a distinct species, a conclusion supported by our study (*G. boninense*: clade E in ITS analysis, clade C in LSU analysis; *G. orbiforme*: clade G in ITS analysis, clade D in LSU analysis). Both species have similar basidiospore size [9-11μm in the Brazilian *G. orbiforme* versus 10-12μm (Núñez & Ryvarden, 2000) and 8.5-12μm (Chang, 1992) in *G. boninense*], and irregular, amyloid cuticle hyphal ends, but resinous layers are present in *G. orbiforme* and apparently absent in *G. boninense* (Chang, 1992; Núñez & Ryvarden, 2000). *Ganoderma orbiforme* was originally described from Guinea in Africa and also recorded in the neotropics, while *G. boninense* was originally described from Bonin Island in Japan and has been reported throughout the Pacific Islands and Sri Lanka, Australia, Taiwan, Japan and China (Chang, 1992; Moncalvo et al., 1995b). Apparently, the geographical distribution of the species was not considered relevant when the synonym was proposed.

Similarly, the Index Fungorum and MycoBank databases consider *G. chalceum* as a synonym for *G. cupreum* (clade E in ITS analysis). Both species have similar basidiospore size [(10-12 × 5-7μm in *G. chalceum* versus 8-11 × 5-7μm in *G. cupreum* (Bresadola, 1911)] and were originally described from western Africa. Contrary to *G. cupreum*, *G. chalceum* has been reported to the neotropics (Ryvarden, 2000; Ryvarden, 2004; Torres-Torres et al., 2012). The sequences of *G. cupreum* are of Australian origin and Smith & Sivasithamparam (2000) commented that more research was needed to verify that the isolates were correctly named. Moreover, there are no sequences of *G. cupreum* and *G. chalceum* originating from the type locality for better comparison.

In this study, we delimit six laccate taxa of *Ganoderma* collected in Brazil based
on rDNA analyses with the support of morphological characters, mostly size of basidiospores, presence/absence of dark, resinous layers in the context, and presence/absence of amyloid hyphal ends in the cuticle. In addition, geographical distribution is also considered relevant as all Brazilian species differ from the previously known laccate *Ganoderma* from Asia, Europe, North and South America.

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**RESUMEN**

Delimitación de algunos *Ganoderma* (Ganodermales): filogenia molecular y morfología. *Ganoderma* incluye especies de gran importancia económica y ecológica, sin embargo, su nomenclatura actual es caótica y poco estudiada en el neotrópico. En este estudio se utilizaron 14 muestras de *Ganoderma* y dos de *Tomophagus* recolectadas en Brasil para la extracción de ADN, amplificación y secuenciación de las regiones ITS y LSU. La delimitación filogenética de seis táxones neotropicales fue discutida con base en especímenes brasileños y secuencias del GenBank. Estas especies mostraron ser distintas de los *Ganoderma* lacados de Asia, Europa, América del Norte y de algunos ejemplares de Argenti- na. Las reconstrucciones filogenéticas confirman que los *Ganoderma* lacados son distintos de *Tomophagus*, aunque pertenecen al mismo grupo. No se confirman los sinónimos de *G. subamboinense* a *G. multipicatum*, de *G. boninense* a *G. orbiforme* y *G. chalceum* a *G. cupreum*. *G. parvulum* se confirma como el nombre correcto para *G. stipitatum*. *G. lucidum* sólo se debe utilizar para especies europeas. Por lo tanto, se propone el uso de nombres publicados válidamente de acuerdo con la distribución geográfica de las muestras, características morfológicas y análisis de ADNr.

**Palabras clave:** Agaricomycetes, taxonomía filogenética, secuencias de ADNr, delimitación de especie, neotrópico.

**REFERENCES**


