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Traditional infrageneric classification of *Gymnopilus* is not supported by ribosomal DNA sequence data

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Abstract: The traditional classification of *Gymnopilus* (Agaricales) recognizes two primary groups, *Annulati* and *Gymnopilus*, based on the presence or absence of a membranous partial veil. While our analyses of DNA sequence data from the nuclear ribosomal ITS1–5.8S-ITS2 (ITS) gene supports the monophyly of the genus, these traditional subgroups were not recovered. Five well-supported clades within the genus were identified through these analyses: 1) the spectabilis-imperialis group; 2) nevadensis-penetrans group; 3) a clade formed by *G. underwoodii*, *G. validipes* and *G.* cf. *flavidellus*; 4) aeruginosus-luteofolius group; and 5) lepidotus-subearlei group. Relationships among these subgroups were not resolved.

Key words: Agaricales, *Annulati*, Cortinariaceae, ITS, phylogeny, Strophariaceae

INTRODUCTION

The genus *Gymnopilus* P. Karst. represents an important component of fungal biodiversity on wood containing more than 200 lignicolous species. *Gymnopilus* has been treated as a member of the family Cortinariaceae *sensu* Singer (1986) or Strophariaceae *sensu* Kühner (1980) in the Agaricales. The genus is well characterized macromorphologically (Hesler 1969, Singer 1986). Hesler (1969) monographed the North American species of the genus. *Gymnopilus* also has been studied in México by Guzmán-Dávalos and Guzmán (1986, 1991, 1995) and Guzmán-Dávalos (1994, 1995, 1996a), in Europe by Høiland (1990), Orton (1993) and Bon and Roux (2002), in Central America by Guzmán-Dávalos (1996b) and Guzmán-Dávalos and Ovrebo (2001), in Zimbabwe by Høiland (1998), and in Australia by Rees and Ye (1999) and Rees et al (1999, 2002).

Gymnopilus, as genus *Fulvidula* Romagn., first was divided into two groups, *Annulatae* Romagn. and *Cortinatae* Romagn., by Romagnesi (1942). *Annulatae* contained species with a persistent, membranous annulus or ''cortina abundantly developed so as to form a distinct annular zone'' (Singer 1986). *Cortinatae* contained species with an arachnoid veil (cortina) or no veil. Singer (1986) accepted the two sections of Romagnesi, naming the latter one *Gymnopilus*. Hesler (1969) also accepted these groups but under the taxonomic rank of subgenera. He divided subgenus *Gymnopilus* into two sections: *Microspori* Hesler and *Gymnopilus*. Later, Guzmán-Dávalos (1995) proposed another section in subgenus *Gymnopilus*, *Macrospori* Guzm.-Dáv. to accommodate large-spored species lacking an annulus.

Most authors have accepted the classification of the genus into two groups (subgenera or sections). However, it often is difficult to assign some species to one or the other group because the annulus or membranous veil is sometimes ephemeral or very easily lost if the specimen is not handled with care. Also, either an annulus or a cortinate veil has been observed on the stipe in different specimens from the same species.

Few molecular studies have included species of *Gymnopilus*, and only one has focused exclusively on the genus. *Gymnopilus sapineus* (Fr.) Maire was used as outgroup in a phylogenetic study of *Cortinarius* using ITS sequences (Høiland and Holst-Jensen 2000). Moser et al (2001) used ITS sequences to establish the phylogenetic relationships of a new spe-

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cies of *Gymnopilus*, *G. turficola* M. M. Moser & H. Ladurner, including in their analysis *G. decipiens* (W. G. Smith) P. D. Orton, *G. luteofolius* (Peck) Singer, *G. odini* (Fr.) Kühner & Romagn., *G. penetrans* (Fr. : Fr.) Murrill, *G. picreus* (Pers. : Fr.) P. Karst., *G. sapineus* and *G. spectabilis* (Fr.) A. H. Sm. Peintner et al (2001) used *Gymnopilus* (*G. penetrans*, *G. sapineus* and *G. spectabilis*) as outgroup in a paper on the multiple origins of sequestrate fungi related to *Cortinarius*. Moncalvo et al (2002), in their molecular study on the relationships among euagarics, found a gymnopiloid clade that included *Gymnopilus* and *Galerina paludosa* (Fr.) Kühner. A gymnopilus clade represented by *G. aeruginosus* (Peck) Singer, *G. spectabilis*, *G. junonius* (Fr.) P. D. Orton, *G. penetrans* and *Hebelomina neerlandica* Huijsman was nested within this larger clade. Thomas et al (2002) also included *Gymnopilus* (*G. aeruginosus*, *G. penetrans*, *G. picreus*, *G. sapineus* and *G. spectabilis*) to establish the relationships of a new genus, *Anamika* K. A. Thomas, Peintner, M. M. Moser & Manim., which is closely related to *Hebeloma*. The only work focused exclusively on *Gymnopilus* is by Rees et al (2002), in which they report on the relationships among Australian and Northern Hemisphere *Gymnopilus* species using sequences of the ITS. Høiland (1990) undertook the only cladistic analysis based on morphological data using the known species of *Gymnopilus* from Norway.

In this paper we use sequence data from the nuclear ribosomal ITS1–5.8S-ITS2 (hereafter referred to as ITS) to test the monophyly of the genus and whether the traditional classification of the genus into two groups based on the presence or absence of an annulus is natural.

MATERIAL AND METHODS

The species included in this study encompass the morphological variation of the genus, with special focus on the subgenus *Annulati*. Specimens were selected to provide broad taxonomic sampling based on the availability of well-preserved material. Some species are known only from the type specimens, so in these cases permits were obtained from curators to use small portions of these specimens for DNA extraction. The familial concepts of both Singer (1986) and of Kühner (1980) were employed because representatives from the families Cortinariaceae (*Cortinarius*, *Dermocybe*, *Galerina*, *Pyrrhoglossum*) and Strophariaceae (*Pholiota*, *Psilocybe*) were included as outgroups. Most of the material used in this study was from old (more than 10 yr) or very old (more than 50 yr) herbarium specimens because few recently collected specimens (i.e., 1 year old or less) were available. DNA extraction was not successful with many of the old and very old herbarium specimens. Only one sample (*G.* cf. *subearlei* 172) was from mycelium grown on MEA (malt-extract agar, Difco). Specimens included in this study are listed in TABLE I. Herbarium acronyms are cited as from Holmgren et al (1990).

DNA extraction.—DNA was extracted at the Departamento de Biología Celular y Molecular, Universidad de Guadalajara, México, using one of these procedures: phenol method of Raeder and Broda (1985), CTAB method of Gardes and Bruns (1993) or employing an extraction kit (Nucleon PhytoPure, Amersham). DNA extraction tests were made to determine the best extraction protocol for obtaining DNA from the minimum amount of material (Santerre et al in preparation). A small part (ca 4 mg) of the pileus, including cutis, context and lamellae, was taken. When possible (e.g., in the case of large basidioma) the cutis was excluded to avoid contamination from exotic spores or material deposited on the surface of the pileus. In very few cases, when the pileus was in poor condition, the sample was taken from the stipe. DNA concentration was determined by spectrophotometry. The DNA extracts were diluted 1:2, 1:5 and 1:10 or were used undiluted in PCR reactions.

PCR amplification.—Polymerase chain reaction (PCR) was performed to amplify the internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene and the internal transcribed spacer 2 (ITS2) following the protocols of Miadlikowska and Lutzoni (2000), with some modifications. Each 50 μL PCR reaction contained 32.7 μL of sterile double-distilled water, 5 μL of 10× reaction buffer (100 μm Tris, 500 μm KCl) with $MgCl_2$ (Behringer-Mannheim), 5 μL of 8 mM dNTPs, 2 units of Taq DNA polymerase (Behringer-Mannheim or Roche), 2.5 μL of each 10 μM primer, 1 μL of BSA (bovine serum albumine) (New England Bio Labs), and 1 μL of DNA template. Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech) also were used, following the manufacturers' protocols to amplify some taxa. Negative controls, without DNA template, were included to detect contamination in the reagents. Primer pairs ITS1F-ITS4, ITS1-ITS4, ITS1-ITS4S or ITS5-ITS4 were used to amplify the entire ITS; ITS1F-ITS2, ITS1-ITS2 or ITS5-ITS5.8S to amplify the ITS1; and ITS3-ITS4, ITS3-ITS4S or ITS5.8SR-ITS4S to amplify the ITS2 (Vilgalys and Hester 1990, White et al 1990, Gardes and Bruns 1993, Kretzer et al 1996). Two primers specific for *Gymnopilus* were designed, ITS1G (5′-CGTAACAAGGTTTCCGTAGG-3′) and ITS4G (5′-GATATGCTTAAGTTCAGCGGG-3′), and the primer pairs used were ITS1G-ITS4G, ITS1G-ITS2 or ITS3-ITS4G.

PCR amplifications were performed in a MJ Research PTC 200 thermocycler. The DNA was denatured at 95 C for 3 min, except for the PCR Beads (93 C for 7 min). Twenty-five cycles of denaturation at 95 C for 1 min, annealing at 50 C for 45 s, and extension at 72 C for 2 min were followed by 15 cycles of 95 C for 1 min, 50 C for 45 s and 72 C for 2 min increasing 5 s each cycle with an extension step of 72 C for 10 min and final incubation at 4 C. Amplification products were visualized by electrophoresis in 1.5% TAE agarose gels (NuSieve, FMC Bioproducts), containing ethidium bromide (1 mg/ml). When necessary, reamplifications were conducted using the above protocol except template DNA was added from either 1 μL of the original PCR product or from a melted punch from the agarose gel.

TABLE I. *Gymnopilus* and outgroup specimens used in this study

No.	Species	Subgenus and section or family for outgroups[1]	Collector, number and herbarium	Locality	Date of collection	GenBank No.
37	*G. aeruginosus* (Peck) Singer	*Annulati*	P. Kroeger 1336 (DAOM-210322)	Canada	Oct. 16, 1988	AY280974
60	*G. aeruginosus* (Peck) Singer	*Annulati*	SAR-88/431 (WTU)	USA	Feb. 2, 1988	AY280975
71	*G. aeruginosus* (Peck) Singer	*Annulati*	F. M. Brigham s.n. (DAOM-137158)	Canada	Aug. 30, 1971	AY280976
88	*G. aeruginosus* (Peck) Singer	*Annulati*	I. Bartelli 200 (F-1116741)	USA	Sept. 9, 1960	AY280977
31	*G. cerasinus* (as *Pholiota cerasina* Peck)	*Annulati*	Herb. E. A. Burt (S)		Sept. 10, 1897	AY280978
39	*G. dilepis* (Berk. & Broome) Singer	*Annulati*	R. Treu (IMI-370900)	Malasia	1996	AY280980
117	*G.* cf. *flavidellus* Murrill	*Gymnopilus, Gymnopilus*	J. Murphy 2452 (F-1116344)	USA	Oct. 14, 1996	AY280981
178	*G. fulvosquamulosus* Hesler	*Annulati*	D. Guravich 220 (MICH-42155)	USA	Nov. 4, 1972	AY280982
89	*G. hispidellus* Murrill	*Annulati*	Standley 53856 (F-1112036)	Honduras	March 20, 1928	AY280983
45	*G.* cf. *hispidellus* Murrill	*Annulati*	S. Garcia s.n. (ENCB)	Mexico	Sept. 21, 1962	AY280984
20	*G. hispidus* (Massee) Murrill	*Annulati*	D. N. Pegler 3254 [K (M) 75211]	Dominica	Nov. 8, 1977	AY280985
119	*G. imperialis* (Speg.) Singer	*Annulati*	Gómez 18197 (F-1051880)	Costa Rica	July 11, 1982	AY280986
28	*G. junonius* (Fr.) P. D. Orton	*Annulati*	L. Guzmán-Dávalos 8220 (IBUG)	France	Nov., 1999	AY280987
30	*G. junonius* (Fr.) P. D. Orton	*Annulati*	L. Guzmán-Dávalos 8222 (IBUG)	France	Nov., 1999	AY280988
1	*G. lepidotus* Hesler	*Gymnopilus, Gymnopilus*[2]	G. Guzmán 30374 (XAL)	Mexico	Sept. 14, 1991	AY280989
6	*G. lepidotus* Hesler	*Gymnopilus, Gymnopilus*	L. Guzmán-Dávalos 7868 (IBUG)	Mexico	1999	AY280990
101	*G. lepidotus* Hesler	*Gymnopilus, Gymnopilus*	G. Guzmán 30602 (XAL)	Mexico	June 6, 1991	AY280991
22	*G. luteofolius* (Peck) Singer	*Gymnopilus, Gymnopilus*[3]	L. R. Hesler & H. Ford (DAOM 80626) (dupl. TENN 15085)	USA	Oct. 25, 1942	AY280992
GB	*G. luteofolius* (Peck) Singer	*Gymnopilus, Gymnopilus*				AF325668
152	*G. medius* Guzm.-Dáv.	*Gymnopilus, Macrospori*[4]	J. García-Franco s.n. (IBUG-Isotype)	Mexico	April 19, 1990	AY280994
153	*G. nevadensis* Guzm.-Dáv. & Guzmán	*Gymnopilus, Gymnopilus*[4]	M. L. Fierros 568 (IBUG)	Mexico	Aug. 3, 1994	AY280995
18	*G.* cf. *palmicola* Murrill	*Annulati*	M. H. Zoberi 342 [K (M) 75214]	Nigeria	1968	AY280979
47	*G. pampeanus* (Speg.) Singer	*Annulati*	J. H. Ross 3975 (MEL-2035632)	Australia	Dec. 29, 1996	AY280996
49	*G. pampeanus* (Speg.) Singer	*Annulati*	S. H. Lewis 401 (MEL-2046438)	Australia	April 30, 1998	AY280997
102	*G. peliolepis* (Speg.) Singer	*Annulati*	O. Röllin 89-16 (IBUG)	Switzerland	July 8, 1989	AY280998
2	*G. penetrans* (Fr.: Fr.) Murrill	*Gymnopilus, Gymnopilus*	L. Guzmán-Dávalos 8196 (IBUG)	Switzerland	Oct. 29, 1999	AY280999
26	*G. penetrans* (Fr.: Fr.) Murrill	*Gymnopilus, Gymnopilus*	L. Guzmán-Dávalos 8223 (IBUG)	France	Nov. 19, 1999	AY281000
36	*G. penetrans* (Fr.: Fr.) Murrill	*Gymnopilus, Gymnopilus*	L. Guzmán-Dávalos 8219 (IBUG)	France	Nov. 19, 1999	AY281001
150	*G. penetrans* (Fr.: Fr.) Murrill	*Gymnopilus, Gymnopilus*	L. Guzmán-Dávalos 8215 (IBUG)	Holland	Nov. 13, 1999	AY281002
104	*G. picreus* (Fr.) P. Karst.	*Gymnopilus, Gymnopilus*	T. Ahti 52192 (H, IBUG)	Finland	Sept. 16, 1994	AY281003
GB	*G. picreus* (Fr.) P. Karst.	*Gymnopilus, Gymnopilus*				AF325661
48	*G.* cf. *punctifolius* (Peck) Singer	*Microspori, Gymnopilus*	M. Evers & D. Sieger (L. Norvell 92-04.20-1) (WTU)	USA	April 20, 1992	AY280993

TABLE I. Continued

No.	Species	Subgenus and section or family for outgroups[1]	Collector, number and herbarium	Locality	Date of collection	GenBank No.
113	*G. robustus* Guzm.-Dáv.	*Annulati*[5]	C. L. Overbo 3528	Panama	May 25, 1997	AY281004
114	*G. robustus* Guzm.-Dáv.	*Annulati*	C. L. Overbo 3509	Panama	May 27, 1997	AY281005
21	*G.* cf. *rugulosus* R. Valenz., Guzmán & J. Castillo	*Annulati*[4]	R. N. Verma M130 [K (M) 75159]	India	July 11, 1984	AY281006
52	*G. sapineus* (Fr.) Maire	*Gymnopilus, Gymnopilus*	I. Kytövuori 90-2488 (H)	Finland	Oct. 9, 1990	AY281007
GB	*G. sapineus* (Fr.) Maire	*Gymnopilus, Gymnopilus*				AJ236079
24	*G. spectabilis* (Fr.) A. H. Sm.	*Annulati*	L. Guzmán-Dávalos 8221 (IBUG)	France	Nov., 1999	AY281008
50	*G. spectabilis* (Fr.) A. H. Sm.	*Annulati*	E. Albertó s.n. (BAFC-32.321)	Argentina	April 21, 1991	AY281009
100	*G. spectabilis* (Fr.) A. H. Sm.	*Annulati*	T. Ahti 49093 (H, IBUG)	Finland	Sept. 27, 1990	AY281010
108	*G. spectabilis* (Fr.) A. H. Sm.	*Annulati*	L. Guzmán-Dávalos 8216 (IBUG)	Belgium	Nov. 17, 1999	AY281011
110	*G. spectabilis* (Fr.) A. H. Sm.	*Annulati*	M. A. Pérez de Gregorio s.n. (IBUG)	Spain	Oct. 3, 1999	AY281012
GB	*G. spectabilis* (Fr.) A. H. Sm.	*Annulati*				AF325662
127	*G. subearlei* R. Valenz., Guzmán & J. Castillo	*Annulati*[4]	G. Guzmán 11648-A (ENCB) Type	Mexico	July 11, 1974	AY281013
172	*G.* cf. *subearlei* R. Valenz., Guzmán & J. Castillo (strain)	*Annulati*	L. Guzmán-Dávalos 7438 (IBUG)	Mexico	Aug. 16, 1998	AY281014
53	*G. suberis* (Maire) Singer	*Annulati*	M. A. Pérez-de-Gregorio s.n. (IBUG)	Spain	Oct. 22, 1999	AY281015
5	*G. subpurpuratus* Guzm.-Dáv. & Guzmán	*Annulati*[4]	L. Guzmán-Dávalos 5303 (IBUG)	Mexico	Aug. 2, 1991	AY281016
151	*G. underwoodi* (Peck) Murrill	*Gymnopilus, Microspori*	L. Guzmán-Dávalos 6248 (IBUG)	Guatemala	Aug. 9, 1992	AY281017
138	*G. validipes* (Peck) Hesler	*Annulati*	M. A. Vincent 6403 (ENCB)	USA	Oct. 2, 1993	AY281018
14	*Gymnopilus* sp.	*Annulati*	B. M. Spooner 290 [K (M) 75158]	Borneo	Nov. 30, 1985	AY281019
GB	*Cortinarius atrovirens* Kalchbr.	Cortinariaceae				AF062619
GB	*Dermocybe phoenicea* (Maire) M. M. Moser	Cortinariaceae				U56055
144	*Galerina autumnalis* (Peck) A. H. Sm. & Singer	Cortinariaceae	L. Guzmán-Dávalos 5246 (IBUG)	Mexico	Oct. 15, 1990	AY281020
145	*Galerina clavata* (Velen.) Kühner	Cortinariaceae	L. Guzmán-Dávalos 8227 (IBUG)	Great Britain	Nov. 27, 1999	AY281021
3	*Pholiota lenta* (Pers.: Fr.) Singer	Strophariaceae	R. Tuomikoski s.n. (H)	Finland	Oct. 22, 1974	AY281022
159	*Psilocybe cubensis* (Earle) Singer	Strophariaceae	L. Guzmán-Dávalos 5864 (IBUG)	Mexico	Aug. 28, 1994	AY281023
140	*Pyrrhoglossum pyrrhum* (Berk. & M. A. Curtis) Singer	Cortinariaceae	L. Guzmán-Dávalos 8075 (IBUG)	Venezuela	Aug. 29, 1999	AY281024

[1] According to Hesler (1969) or Singer (1986).
[2] According to Guzmán-Dávalos (1995).
[3] Singer (1986) considered it in *Annulati*.
[4] According to Guzmán-Dávalos and Guzmán (1995).
[5] According to Guzmán-Dávalos and Ovrebo (2001).

Sequencing.—Amplification products were excised from 1% TALE agarose gels (NuSieve, FMC Bioproducts), melted at 70 C, and 1 μL of GELase™ Agarose Gel-Digesting Preparation (Epicentre Technologies) was added to each product and incubated at 45 C for at least 1 h. Sequencing reactions were performed with BigDye™ Terminator (ABI Prism, Perkin-Elmer Biosystems) in a 10 μL final volume, following manufacturers protocols and using the same primers as in the PCR reactions. Depending on the automated sequencer used, one of these protocols was used to precipitate the products: 1) 10 μL of deionized sterile water, 2 μL of 3 M NaOAc and 50 μL of 95% EtOH; or 2) 30 μL of de-ionized sterile water and 60 μL of isopropanol. Sequences were obtained either by polyacrylamide gel electrophoresis performed on an ABI 377A automated DNA sequencer (Perkin-Elmer, Applied Biosystems) or by capillary electrophoresis on an ABI-Prism 3100 Genetic Analyzer (Perkin-Elmer, Applied Biosystems).

Assembly of sequence fragments, correction of raw sequences and alignment of consensus sequences were carried out with Sequencher 3.0 (Gene Codes Corp.). Every sequence was subjected to a BLAST search in GenBank and doubtful sequences (the ones that did not blast to *Gymnopilus*) were removed. Fifty-three new sequences were generated and seven were retrieved from GenBank. Alignments were checked by eye and manually corrected when necessary using MacClade 4.0 (Maddison and Maddison 2000). New sequences have been submitted to GenBank and alignments are deposited in TreeBASE (matrix accession numbers M1433, M1434; study accesion number S883).

Phylogenetic analyses.—A total of 57 sequences from 38 taxa, which included six sequences from GenBank (*Gymnopilus luteofolius*, *G. picreus*, *G. sapineus*, *G. spectabilis*, *Cortinarius atrovirens*, *Dermocybe phoenicea*), were used in the analyses (TABLE I). Two datasets were examined: one which used the full 57 specimen matrix and another which used all ingroup sequences but restricted the outgroup to the single most closely related taxon, *Galerina autumnalis*, based on analyses of the first dataset. Maximum-parsimony (MP) analyses were performed on both sets, while maximum-likelihood (ML) and Bayesian methods were implemented only on the set of 51 sequences. The MP and ML analyses were performed with PAUP* 4.0b10 (Swofford 2000), while MrBayes 2.01 (Huelsenbeck and Ronquist 2001) was used to perform the Bayesian analysis.

Equally weighted and unequally weighted MP analyses were performed. In the equally weighted MP analyses, ambiguous regions due to gaps were excluded. In the unequally weighted MP analyses, changes among transitions/transversions were subjected to a specific symmetric step matrix with costs among changes calculated as the negative natural logarithm of their relative frequencies using StepMatrix 2.1 (François Lutzoni and Stefan Zoller, Department of Biology, Duke University). The ambiguously aligned regions also were included as unequivocally coded characters using the program INAASE 2.3b (Lutzoni et al 2000) to recode these regions. Each coded character was subjected to a specific symmetric step matrix derived from pairwise comparisons of sequences, accounting for the optimal number of changes between all possible combinations of any two sequences. Gaps were treated as missing characters in all MP analyses.

Heuristic searches were conducted under these conditions: starting trees obtained by stepwise addition, random addition sequence with 1000 replicates, tree-bisection-reconnection (TBR) as the branch swapping algorithm, branches collapsed if maximum branch length is zero, and MulTrees option in effect. In some analyses the option to limit branch swapping to 5 or 10 million rearrangements per replicate was implemented. This procedure was necessary due to the large number of equally most-parsimonious trees generated from the inclusion of identical sequences that could not be resolved. Support for nodes recovered from MP analyses was obtained from 1000 bootstrap replications (Felsenstein 1985). The conditions were the same as above, except that the number of random-addition replicates was set to 10 and in some cases branch swapping was limited to 1 million rearrangements per replicate.

For ML and Bayesian analyses, the most likely model of evolution was determined through nested likelihood ratio tests as implemented in Modeltest 3.06 (Posada and Crandall 1998). In both analyses, the ambiguously aligned characters were excluded and the constant characters were included. The ML analysis was performed with 100 random-addition replicates, while the Bayesian analysis was run for 10 million generations with trees sampled every 1000th generation. The first 2000 trees representing the burn-in phase of the analysis were discarded, and posterior probabilities were calculated from a consensus of the remaining 8000 trees. This analysis was repeated five times, starting from random trees to ensure the same set of trees were being sampled during each analysis.

RESULTS

The final alignment for the 57 ITS sequences consisted of 800 nucleotide positions after the introduction of gaps. A total of 24 ambiguously aligned regions were found. There were 51 informative characters with the ambiguous regions excluded and 75 informative characters when the recoded ambiguous regions were included.

The equally weighted parsimony analyses, excluding the ambiguously aligned regions, were possible to run in a branch-and-bound search due to the small number of informative characters. For the rest of the parsimony analyses, the only option available due to the size of the data matrix was heuristic searches. Because the results of the first analyses with the 57 taxa dataset documented that *Galerina autumnalis* was the closest outgroup to *Gymnopilus*, subsequent analyses were undertaken with only this taxon as outgroup. Excluding the more-distant outgroups reduced the number of gaps and correspondingly, the size and number of ambiguously aligned regions, which improved the alignment and increased the number of parsimony-informative characters.

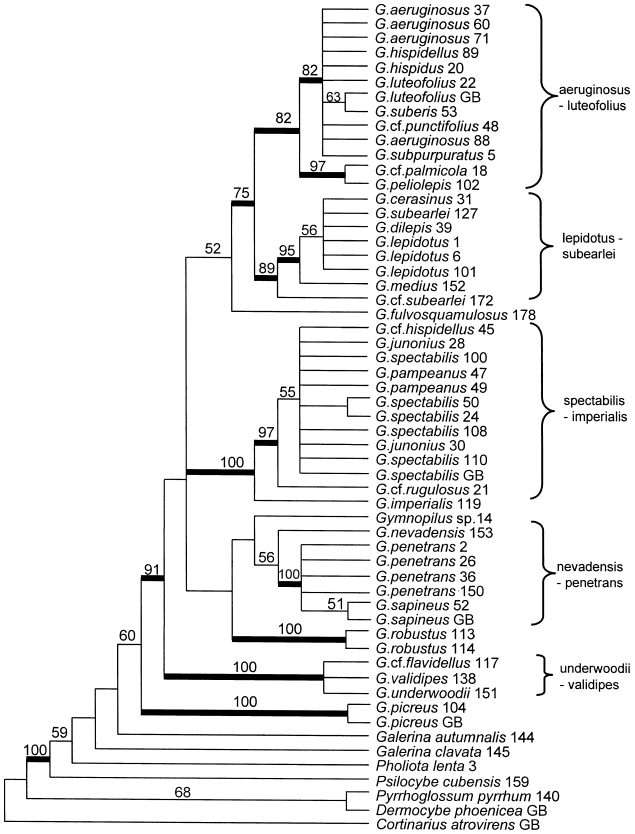

FIG. 1. Strict consensus of 76 840 most-parsimonious trees (tree length = 649 steps, CI excluding uninformative characters = 0.72, RI = 0.86, RC = 0.62) of 50 specimens of *Gymnopilus* and seven outgroups, based on ITS sequence data, unequally weighted and with ambiguous regions unequivocally coded (informative characters = 75). Bootstrap values >50% obtained from 1000 replications are given above each branch. Highly supported clades (bootstrap support ≥70%) are indicated by thickened branches.

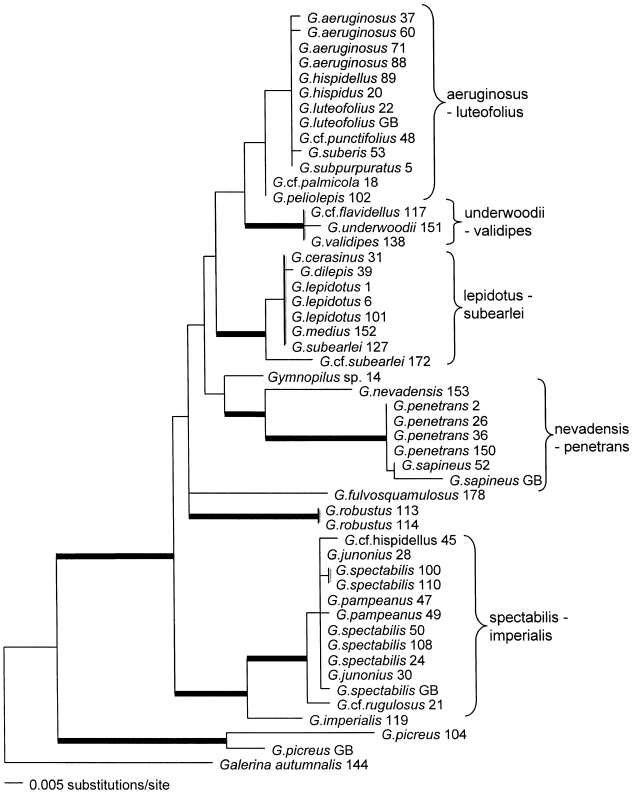

FIG. 2. One of two phylograms resulting from the maximum likelihood analysis (−ln L = 1622) of 51 samples of *Gymnopilus*, with *Galerina autumnalis* as outgroup, based on ITS sequence data, and implementing the HKY+G model. Thickened branches represent posterior probabilities above 95% determined through Bayesian analysis.

Equally weighted parsimony analysis of the 57 sequences with a branch-and-bound search resulted in a tree with low bootstrap support for many of the branches (not shown). The heuristic search with transitions/transversions unequally weighted, plus the ambiguous regions unequivocally coded, resulted in 76 840 trees, with a tree-length of 649 steps. The consistency index (CI) excluding uninformative characters was 0.72, homoplasy index (HI) = 0.28, retention index (RI) = 0.86, and rescaled consistency index (RC) = 0.62. The strict-consensus tree (FIG. 1) has the same general topology as the one that resulted from the branch-and-bound search but with higher bootstrap values. The *Gymnopilus* clade is present (FIG. 1), although with only 60% bootstrap support. Excluding *G. picreus*, the remaining *Gymnopilus* species form a well-supported clade, with a bootstrap of 91%.

The dataset of 51 sequences (representing 32 taxa), with *Galerina autumnalis* as outgroup, resulted in 781 characters and 22 ambiguous regions. Seventy-three parsimony-informative characters were present, with the ambiguous regions excluded, and 95 when the recoded ambiguous regions were included. The unequally weighted analyses resulted in 2581 most-parsimonious trees of 462 steps (not shown), with CI excluding uninformative characters = 0.69, HI = 0.31, RI = 0.88 and RC = 0.60.

One of the two trees obtained in the maximum-likelihood analysis is shown in FIG. 2 (−ln L = 1622). These trees differed only in that *G. spectabilis* and *G. junonius* grouped together in one of them. The most likely model of evolution determined through nested likelihood ratio tests was HKY+G (Hasegawa et al 1985, Posada and Crandall 1998). The number of substitution types was two, the transition/transversion ratio was 3.8307, the proportion of invariable sites was zero and the gamma shape parameter was 0.1848. Node support is indicated by posterior probabilities determined through Bayesian analysis. The same major clades were recovered by ML analysis as were

identified in the MP trees; however, relationships among the clades differ (FIGS. 1 and 2).

Neither the division of the genus into *Annulati* and *Gymnopilus* nor recognition of the three sections in subgenus *Gymnopilus* was supported because the species belonging to these groups occurred in multiple clades in all trees. Although ITS data did not fully resolve infrageneric relationships, five well-supported clades in the genus were recognized with bootstrap values above 80% and posterior probabilities over 95% (FIGS. 1 and 2). These clades and their support values (bootstrap and posterior probability, respectively) are: 1) the aeruginosus-luteofolius group (82%, <95%); 2) the lepidotus-subearlei group (89%, 100%); 3) the spectabilis-imperialis group (100%, 100%); 4) the penetrans-sapineus group (100%, 100%), or including *G. nevadensis* (the nevadensis-penetrans group) (56%, 100%); and 5) the underwoodii-validipes group (100%, 100%).

DISCUSSION

ITS sequence data have been widely used to try to resolve phylogenetic relationships of fungi at the infrageneric level. However, in many cases ITS sequences among species in a genus are extremely variable because insertions and deletions are common. This problem exists even in closely related species, making alignments problematic (e.g., Hsiang and Wu 2000). In other cases, ITS sequences show low variation, being very similar or identical among investigated taxa (e.g., Johannesson et al 2000). Both of these conditions can result in the ITS providing few parsimony-informative characters, leading to low resolution among the investigated taxa. The number of informative characters found among the *Gymnopilus* taxa in this study (51/75 for 38 taxa in 57 samples, or 73/95 for 32 taxa in 51 samples, ambiguous regions excluded/unequivocally coded) is relatively low compared to the numbers reported for some groups of fungi, such as *Ceratobasidium* and *Thanatephorus* where González et al (2001) found 162 phylogenetic informative characters for 28 anastomosis groups in 122 accessions. However, the same relative number of informative characters, or fewer, that were uncovered in our analyses has been reported in studies of other genera. For example, in *Antrodiella*, Johannesson et al (2000) reported 34 informative characters for 12 taxa among 30 sequences, Wu et al (2000) reported 70 informative characters for 18 taxa of *Suillus* among 40 samples, while Moser and Peintner (2002) found 58 informative characters for 11 taxa of *Cortinarius* among 23 sequences.

The genus Gymnopilus.—The ITS sequence data provided sufficient resolution to consider the genus monophyletic, although there is no bootstrap or posterior probability support for including *G. picreus* in the genus. The limits of the genus are difficult to circumscribe based solely on morphology. The differences among some species of *Gymnopilus* and *Galerina* are obscure (Singer 1986, Horak 1989, Rees et al 1999), with the presence of styrylpyrone pigments in *Gymnopilus* (e.g., Hatfield and Brady 1968, Dangy-Caye and Arpin 1974, Gill and Steglich 1987, Høiland 1990) but lacking in *Galerina*, the only reliable feature that separates them, according to Rees et al (1999). *Gymnopilus picreus* always has been considered a *Gymnopilus*, although the color (''red-brown to almost chestnut-brown'', Høiland 1990) and consistency of the basidioma (somewhat cartilaginous, especially the stipe), suggest a relationship with *Galerina* or *Phaeocollybia*. The presence of styrylpyrones in *G. picreus* was demonstrated by Høiland (1990), and he selected it as the lectotype of the genus. Rees et al (1999) and Bon and Roux (2002) did not accept Høiland's lectotypification and recognize *G. liquiritiae* (Pers. : Fr.) P. Karst. the type species. Moncalvo et al (2002) identify a gymnopiloid clade consisting of four species of *Gymnopilus* (*G. picreus* was not included in the analyses), *Hebelomina* and *Galerina paludosa* among the 117 clades of euagarics uncovered in their analysis of nuclear ribosomal large-subunit (nrLSU) data. However, *Galerina marginata* (Batsch) Kühner and *Galerina nana* (Petri) Kühner were resolved close to the panaeolideae clade, indicating that the genus *Galerina* is polyphyletic. *Galerina marginata* is very closely related to *G. autumnalis* (Smith and Singer 1964), which was one of our outgroup species, or is an earlier synonym of that name (Gulden et al 2001). Moser et al (2001) and Thomas et al (2002) resolved *G. picreus* basal to the other species of *Gymnopilus* yet still inside *Gymnopilus*, but they did not include species of *Galerina* in these analyses. Rees et al (2002) concluded that *Gymnopilus* is monophyletic, but with very low bootstrap support of 57% and a decay value of 2, when it includes *Galerina eucalyptorum* E. Horak and *Pyrrhoglossum pyrrhum*. However, we found *P. pyrrhum* outside the *Gymnopilus* clade and more closely related to *Dermocybe phoenicea* (FIG. 1).

Kühner (1980), placing a strong value on chemical characters, grouped *Gymnopilus*, *Galerina*, *Pholiota* and others in the Strophariaceae because of the presumed presence of styrylpyrones. However no styrylpyrones have been found in any of the tested species of *Galerina* (Rees et al 1999). Høiland and Holst-Jensen (2000) found that the ITS sequence of *Gymnopilus sapineus* was more similar to that of *Hypholoma capnoides* (Fr.) P. Kumm. (Strophariaceae) than to

Cortinarius. Results from our study support those of Høiland and Holst-Jensen (2000), with *Psilocybe cubensis* (= *Stropharia cubensis*), *Pholiota*, *Galerina* and *Gymnopilus* forming a clade with a bootstrap value of 100% (FIG. 1) distinct from *Pyrrhoglossum*, *Dermocybe* and *Cortinarius*.

Infrageneric relationships.—Our phylogenetic analyses of ITS sequences did not recover sufficient information to completely resolve infrageneric relationships in *Gymnopilus*. However, five well-supported clades were identified. Relationships among these clades varied among the trees obtained during our separate analyses. However, because there is no support for the backbone in any of these trees, these differences might not be significant.

Some genera, e.g., *Amanita* (Drehmel et al 1999), have many morphological characters that have been used to support infrageneric groups. Unfortunately, this is not the case for *Gymnopilus*, where only the presence or absence of a partial membranous veil and the size of the basidiospores have been used. Both characters have been shown to be highly homoplastic and of little value at this taxonomic level.

The two subgenera or sections considered by Hesler (1969) and Singer (1986) were not supported by our analyses. Singer (1951), discussing the split of the genus into the two groups, noted, "the veil may not in all cases be a character of primary importance." The three sections in subgenus *Gymnopilus* also were not supported. Section *Gymnopilus* was represented in this study by *G.* cf. *flavidellus*, *G. luteofolius*, *G. nevadensis*, *G. penetrans* (considered as synonym of *G. sapineus* by some authors, including Høiland [1990]), *G. picreus* and *G. sapineus*. Section *Macrospori* was represented by *G. medius*, and section *Microspori* by *G.* cf. *punctifolius* and *G. underwoodii*. The latter species was a questionable member of *Microspori* because its spores are relatively large (6–7 μm *sensu* Hesler 1969). Section *Microspori* was defined with spores 3.5–7 μm long (Hesler 1969). The material used in our study had spores 6.4–8 μm long (Guzmán-Dávalos 1996b). We unsuccessfully attempted to extract and sequence DNA from other species from this section. Many species from section *Microspori* have small basidiomata, and it seems that there is some relation between the size of the basidiomata and the success in DNA extraction in *Gymnopilus*, as already noticed by B. Rees (pers comm). *Gymnopilus lepidotus* was included in section *Microspori* by Hesler (1969) but was transferred to section *Gymnopilus* by Guzmán-Dávalos (1995) due to the size of the spores. The taxon is now considered in subgenus *Annulati* (Guzmán-Dávalos, unpubl data) because of the presence of a thick arachnoid veil that sometimes develops as a submembranous annulus.

Gymnopilus robustus Guzm.-Dáv. is another species that is difficult to place in either subgenus. The type specimen fits the circumscription of subgenus *Gymnopilus*, section *Macrospori* because it displays a fibrillose, evanescent partial veil and has large basidiospores (Guzmán-Dávalos 1995). Recently collected specimens, however, showed that the species could have basidiomata with a submembranous veil, placing it in subgenus *Annulati* (Guzmán-Dávalos and Ovrebo 2001). Our analyses did not completely resolve the affinities of this species, but it is clear that *G. robustus* is not part of the spectabilis-imperialis clade, as was anticipated due to its large and annulated basidioma. *Gymnopilus robustus* is resolved distant from the spectabilis-imperialis group in both the MP (FIG. 1) and ML trees (FIG. 2).

The groups identified in this study are described below. Although each of these groups is well supported by the MP, ML and Bayesian analyses (FIGS. 1 and 2), some of them have no obvious morphological synapomorphies that clearly define them.

The aeruginosus-luteofolius clade. All species included here have the pileus covered by purplish to reddish erect squamules, at least when young. They are from temperate to tropical regions. In some of them (*G. aeruginosus*, *G. luteofolius*, *G.* cf. *punctifolius* and *G. subpurpuratus*), the basidioma stains green when bruised or has a greenish-blue or bluish coloration that suggests the presence of psilocybin (Gartz 1984).

The lepidotus-subearlei clade. Except for *G. cerasinus*, which has a fibrillose pileus and a temperate distribution, the species included in this group have erect, reddish squamules in the pileus and are restricted to tropical or subtropical environments. *Gymnopilus subearlei* stains green when bruised. This clade, together with the previous one, is part of a larger clade (75% bootstrap) in the parsimony trees (FIG. 1) that includes species with reddish scales that contain psilocybin (although the latter character state is lost in some species in the overall clade). These two clades also are part of a large nonsupported clade in the ML tree, but in this case the clade also includes the underwoodii-validipes clade, which contains species that are morphologically distinct (FIG. 2).

The spectabilis-imperialis clade. This group contains all the species with large, robust basidioma that have a thick, membranous annulus and fibrillose to slightly squamose pileus, except *G. robustus*. The complex formed by *G. junonius*, *G. pampeanus* and *G. spectabilis*, considered as synonyms by some authors, has species with ellipsoid spores that are longer than 8 μm. Further studies are needed to elucidate if they

represent a single variable species. Singer (1986) suggested that *G. suberis* could be a subspecies of *G. spectabilis*, even though there are obvious differences between them. *Gymnopilus suberis* (Maire 1928) has smaller spores and erect, reddish squamules in the pileus disk of young basidiomata that never are present in *G. spectabilis*. In our analyses, it is clear that *G. suberis* is not nested within the spectabilis-imperialis clade but is related to other species with squamose pilei in the aeruginosus-luteofolius clade, such as *G. subpurpuratus*. *Gymnopilus imperialis* is the basal species of this clade. This species and *G.* cf. *rugulosus* are distinguished from the other species in the clade by having tuberculate and broadly ellipsoid to subglobose basidiospores, rather than ellipsoid and verrucose basidiospores.

The nevadensis-penetrans clade. The three species forming this complex are macromorphologically similar but they display some differences in micromorphology. For example, the pleurocystidia in *G. nevadensis* are larger than the cheilocystidia while in the other two species the pleurocystidia are of similar size or more often, smaller than the cheilocystidia. *Gymnopilus nevadensis* is rare and known only from México, while the other two species are commonly encountered and distributed worldwide. *Gymnopilus penetrans* and *G. sapineus* are very similar to each other both macro- and micromorphologically and occur in similar habitats (i.e., on branches and logs in coniferous or temperate forests). The primary differences between these two species are in the ornamentation of the pileus, being fibrillose and lacking pileocystidia in the former and squamulose with poorly differentiated pileocystidia in the latter. They are grouped together with a 100% bootstrap and posterior probability (FIGS. 1 and 2). Further studies are needed to confirm if they are conspecific or separate species.

The underwoodii-validipes clade. The three species in this clade are known only from North and Central America. *Gymnopilus validipes* closely resembles *G. spectabilis*, as well as the included specimen of *G.* cf. *flavidellus* (the herbarium specimen originally was identified as *G. spectabilis*). *Gymnopilus underwoodii* is a species with medium-size basidioma and morphologically is different from the other two species in the clade. It is known only from the U.S.A. and Guatemala (Guzmán-Dávalos 1996b). This well-supported clade is basal to the core *Gymnopilus* in the parsimony tree (FIG. 1) but is deeply nested in the likelihood tree, resolving close to the aeruginosus-luteofolius clade (FIG. 2).

In conclusion, the genus is monophyletic, although bootstrap and posterior probability support is lacking for recognizing *G. picreus* in *Gymnopilus*. The genus, excluding *G. picreus* has strong support. The traditional subgenera *Annulati* and *Gymnopilus*, as well as the sections of the later, are not supported. Partial veil characters and basidiospore size are highly homoplastic characters. Five well-supported clades were recognized within the genus, but relationships among these clades and for some other taxa were not robustly resolved.

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