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Crinipellis brasiliensis, a new species based on morphological and molecular data

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Abstract: *Crinipellis perniciosa* infects a diversity of hosts causing severe damage to *T. cacao* production in many Brazilian growing regions. We compared isolates of *Crinipellis* from different geographic origins and hosts in Brazil by structural analysis using light (LM) and scanning electronic microscopy (SEM), as well as RFLP and sequence data based on the nuclear rDNA ITS region. Statistical analyses of morphometric data of basidia and basidiospores revealed a distinct group of isolates of *Crinipellis* obtained from *Heteropterys acutifolia* when compared to representatives from *Theobroma cacao, Solanum lycocarpum* and *Heteropterys nervosa.* A similar distinction also was observed based on sequence data of the ITS region such that combined results allowed for the segregation of a new species within the genus *Crinipellis.*

Key words: *Crinipellis perniciosa,* cocoa witches' broom, internal transcribed spacer, phylogeny, ribosomal RNA genes, *Theobroma cacao,* tropical mycology

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INTRODUCTION

The genus *Crinipellis* Pat., originally described by N. Patouillard in 1889 (Singer 1942), currently accommodates 75 species (Kirk et al 2001), the majority from the Western Hemisphere. Among them deserving attention is *Crinipellis perniciosa* (Stahel) Singer, a hemibiotrophic pathogen, causal agent of cocoa (*Theobroma cacao* L.) witches' broom, responsible for losses exceeding 90% of the crop yield in the Amazonian basin and in the state of Bahia (Evans 1981). With the establishment of *C. perniciosa* in the main cocoa growing regions of Brazil (Perreira et al 1989), the country has dropped from second largest producer of cocoa to sixth place in 15 y.

In addition to *T. cacao, C. perniciosa* infects a variety of other families that include the *Bignoniaceae* (Evans 1978, Hedger et al 1987), *Bixaceae* (Purdy and Schmidt 1996), *Malpighiaceae* (Bastos et al 1998, Resende et al 2000), *Solanaceae* (Bastos and Evans 1985), and *Sterculiaceae* (Evans 1978, Bastos et al 1988). Evans and Barreto (1996) were the first to report on *Solamum cernuum* Vell., a native solanaceous species as host for *C. perniciosa* in remnant forest (''Zona da Mata''), state of Minas Gerais, where *T. cacao* was never cultivated.

Pegler (1978) examined a range of South American specimens ascribed to *C. perniciosa* and designated three varieties within the species based on pileal color and basidiospore size: (i) var. *perniciosa*, characterized by a red pileus fading to pink, with margin becoming white as the pileus matures, concentrating at the pileal disk and striae, typified by an specimen from *T. cacao* in Trinidad; (ii) *var. ecuadorensis* (Stahel) Pegler, from *T. cacao* and *Arrabidaea verrucosa* (Standl.) A.H. Gentry (liana), first proposed by Stahel (1924), who noted that basidiomata of *C. perniciosa* from Ecuador were characterized by deeper, more uniformly pigmented pilei but lacking the pale margins seen on basidiomata from Surinam and Trinidad; and (iii) var. *citriniceps* Pegler, with citron yellow basidiomata, probably a mutant variety deficient in pileal pigment synthesis, found on a *T. cacao* broom in Pichilingue, Ecuador, by Evans in 1976. Pegler's concept of *C. perniciosa* with three varieties has been questioned because no mention was made of significant morphological differences between herbarium specimens of basidiomata from *A. verrucosa* and *T. cacao* material from Ecuador (Griffith et al 1994).

Five biotypes of *C. perniciosa* have been described:

(i) Sterculiaceae type (C-biotype), infecting *Theobroma* spp. and *Herrania* spp. (Evans 1978, Bastos et al 1988); (ii) Solanaceae type (S-biotype), restricted to species in the Solanaceae (Bastos and Evans 1985); (iii) Bixaceae type (B-biotype), on host species in the Bixaceae (Purdy and Schmidt 1996); (iv) L-biotype, a saprotroph colonizing a variety of substrates, including bignoniaceous lianas as an endophyte or causing latent infection (Evans 1978, Hedger et al 1987, Griffith and Hedger 1994); and recently (v) H-biotype isolated from a malpighiaceous shrub, *Heteropterys acutifolia* A. Juss. (Griffith at al 2003).

In contrast to Pegler's findings Hedger et al (1987) found two distinct populations of *C. perniciosa* in *T. cacao* and *A. verrucosa*, with basidiomata grown in infected *T. cacao* (C-biotype) different from those in *A. verrucosa* (L-biotype) in Ecuador. The L-biotype basidiomata were significantly larger, darker pigmented, had both stouter basidiospores and cheilocystidia than C-biotype basidiomata. Resende et al (2000) obtained isolates of *C. perniciosa* from *H. acutifolia*, in Minas Gerais, which were considered by the authors as morphologically identical to those from *T. cacao*.

Pathogenic variability of *C. perniciosa* first was described by Wheeler and Mepsted (1988), identifying two groups on *T. cacao*: group A, isolates from Bolivia, Ecuador (Pichilingue) and most isolates from Colombia; and group B, isolates from Brazil, Trinidad-Tobago and Venezuela. Cross inoculations as well as morphological comparisons discriminated four biotypes of *C. perniciosa*: C-biotype, S-biotype, B-biotype and L-biotype (Hedger et al 1987, Bastos et al 1988).

Bastos et al (1988) showed that isolates of *C. perniciosa* from *T. cacao* were pathogenic on *T. cacao*, *T. speciosum* and *Herrania* spp. seedlings but not to *Solanum esculentum*, *S. melongena* and *S. gilo*, which did not show any symptoms. Furthermore L-biotype isolates induced hypertrophy and broom formation only in solanaceous hosts and *Herrania* spp., while *Theobroma* spp. were weakly pathogenic, inciting only swollen buds. Cross inoculation tests (Resende et al 2000) also have shown that an isolate of *C. perniciosa* from *H. acutifolia* caused broom formation in ''Catongo'' cocoa, a susceptible cultivar of *T. cacao*. However they were able to induce only symptoms of hypertrophy in ''Theobahia'' cocoa, a resistant cultivar of *T. cacao*, even when applying a high inoculum concentration. Viana Júnior (2001) also tested the same isolate from *H. acutifolia* at an even higher inoculum concentration and observed only symptoms of hypertrophy on *T. grandiflorum* and *T. cacao* (cv. ''Catongo''). In contrast, Bastos et al (1998) reported that isolates of *Crinipellis* from another *Malpighiaceae*, *Mascagnia* cf. *sepium* Vell., were pathogenic on *T. cacao*.

Analysis on the basis of morphology, growth rates, somatic incompatibility tests (Griffith et al 1994, Griffith and Hedger 1994), molecular comparisons and biochemical tests allowed for the identification and distinction of *C. perniciosa* isolates from different geographic origins (Hedger et al 1987, Bastos et al 1988, Wheeler and Mepsted 1988). Molecular variability in *C. perniciosa* from different areas in the Amazonia and Bahia, using RAPD markers, revealed greater variability within and between regions than among isolates from different host species (Andebrhan and Furtek 1994, Andebrhan et al 1999, Niella et al 2000). Later Gomes et al (2000) using RAPD markers detected geographically defined groups of isolates from *T. cacao* also in Bahia.

The nuclear ribosomal DNA (rDNA) is an important locus for molecular systematic investigations, and a number of phylogenetic studies have been conducted within the *Tricholomataceae*, contributing to the clarification of evolutionary relationships over a wide range of taxonomic levels (Hibbett et al 1997, Lebel and Castellano 2002). Thus Anderson et al (1989) demonstrated that the rDNA repeat in *Armillaria* is informative in terms of phylogeny, based upon restriction mapping. Weiss et al (1998), determined phylogenetic relationships within the genus *Amanita*, based on sequence comparison of the rDNA large subunit in 49 species. Hughes et al (1999) suggested new biological and morphological species within the genus *Flammulina*, and Moncalvo et al (2000), compared families within the *Agaricales*, including *Tricholomataceae,* represented by the genera *Crinipellis* and *Marasmius*. However no phylogenetic data to date is available specifically for *C. perniciosa*.

Thus the aim of this study primarily was to determine whether variability among Brazilian isolates of *Crinipellis* correlates with geographic origin or host based on morphology, determination of genetic variation via RFLP and sequence analysis of the rDNA ITS regions and 5.8S RNA gene. Another objective was to determine whether the differences, morphological and molecular, among isolates would be enough to define a new taxon within the genus *Crinipellis*.

MATERIALS AND METHODS

Morphological studies.—Isolates of *Crinipellis* were obtained from a wide range of host and geographic locations. Multisporic isolates of putative *C. perniciosa* from *Theobroma cacao*, *T. grandiflorum* (Willd. ex Spreng.) Schum (from the Amazon region), *Solanum lycocarpum* A. St Hil. and *Heteropterys acutifolia* examined in this study were provided by Universidade Federal de Lavras (UFLA), in Lavras, Minas Gerais, and Comissão

Executiva do Plano da Lavoura Cacaueira (CEPLAC), in Ilhéus, Bahia. An isolate from *Heteropterys nervosa* A. Juss. was collected in ''Cerradão'' preserved area of Centro de Pesquisa Agropecuária dos Cerrados (CPAC/Embrapa), in Planaltina (Distrito Federal, Brazil). The isolates of *Crinipellis* from *T. grandiflorum* (in Bahia) and *S. paniculatum* Linn. were provided by Fazenda Almirante (FA), Cocoa Research Center, in Itajuípe, Bahia. The individual basidiomata of *Crinipellis* from *T. cacao* were produced in vitro with a standard artificial culture medium described by Griffith and Hedger (1993) and modified by Niella et al (1999). Basidiomata of *Crinipellis* from *S. lycocarpum*, *H. acutifolia* and *H. nervosa* were obtained from infected tissue material (dry fan brooms) after the brooms had been submitted to alternating wet and dry periods (8–12 h) in a moist chamber (Rocha and Wheeler 1985) at 25 ± 2 C with photoperiod of 12 h. All basidiomata were washed in sterile distilled water and dried on sterile filter paper before preparation for light and electron microscopy (LM and SEM) analyses and to remove detritus and other substances (dust and substrate remnants) on the analyzed surfaces. The collections of basidioma were deposited in the Mycological Collection, Herbarium of the University of Brasília (Herbarium UB [Mycol. Col.]).

Single spore cultures were obtained from basidiomata used in the morphological studies. These cultures were prepared by inoculating 2% water agar plates with diluted spore suspensions. Single spore microcolonies were transferred to fresh potato-dextrose agar (PDA) under a binocular microscope following McGreary and Wheeler (1988). In a preliminary study (Arruda et al 2003b) a total of 120 single spore cultures were established, out of which seven representative single spore isolate were used in the current study (CANG/1, BE12/18, UB [Mycol. Col.] 1998; UB [Mycol. Col.] 2021; UB [Mycol. Col.] 2027; UB [Mycol. Col.] 2041; and UB [Mycol. Col.] 2053) along with five multiple spore cultures (UB [Mycol. Col.] 2054; UB [Mycol. Col.] 2055; FA617; and FA619) (TABLE I). All multiple spore isolates are maintained at UFLA, Universidade de Brasília (UnB) and Fazenda Almirante, and all single spore cultures are preserved and deposited at the Departamento de Fitopatologia, UnB.

The light-optical microscopic (LM) analyses (observations and measurements) were made from five basidiomata of *C. perniciosa* isolated from *T. cacao* (Canavieiras, Bahia state, UB [Mycol. Col.] 19192), five basidiomata from *S. lycocarpum* (Lavras, Minas Gerais, UB [Mycol. Col.] 19197), eight basidiomata of *Crinipellis* sp. isolates from *H. acutifolia* (Itumirim, Minas Gerais state, UB [Mycol. Col.] 191980) and five basidiomata of *C. perniciosa* isolates from *H. nervosa* (Planaltina, Distrito Federal, UB [Mycol. Col.] 19193) (TABLE II). These basidiomata were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.8) for at least 24 h at 4 C. Sections were cut with a freezing microtome, model HM 505 E (Micron Laborgeräte, Walldorf, Germany), mostly at a thickness of 16–20 μm, and mounted on a microscopic slide. Measurements were made at 100× magnification with a calibrated optical micrometer. Basidium and spore dimensions are given as: length range × width range. Microphotographic measurements and observations were made from colorless and colored material (stained with cotton blue in lacto-glycerol) using bright field and phase contrast optics, in light microscope (Zeiss-Axiophot E, Carl Zeiss Oberkavhen, Alemanha). All morphological features were described following Singer (1942, 1976). In addition statistical analyses were performed with the SAS program, version 6.12 (SAS Institute INC., Cary, North Carolina) and Tukey's test based on morphometric measurements (length and width) of 50 basidia and 50 basidiospores from each basidioma studied.

For scanning electron microscopy (SEM), 30 basidiomata of *C. perniciosa* from *T. cacao* were analyzed, representing the localities of Santo Amaro (UB [Mycol. Col.] 19187), Ilhéus (UB [Mycol. Col.] 19188), Canavieiras (UB [Mycol. Col.] 19189 and 19199) and Ipiaú (UB [Mycol. Col.] 19190), in Bahia; Ouro Preto (UB [Mycol. Col.] 19192), in Rondônia state; Belém (UB [Mycol. Col.) 19194) in the states of Pará, and Manaus (UB [Mycol. Col.) 19196) in Amazonas. In addition nine basidiomata directly collected from dry brooms from other hosts also were analyzed, three basidiomata from *H. nervosa* (Planaltina, Distrito Federal UB [Mycol. Col.] 19193); three basidiomata from *S. lycocarpum* (Lavras, Minas Gerais UB [Mycol. Col.] 19197); and three basidiomata from *H. acutifolia* (Itumirim, Minas Gerais UB [Mycol. Col.] 19198). Pieces of basidiomata either were fixed in 37% FAA (formaldehyde acid acetic alcohol) or in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.8) at least 24 h at 4 C and subsequently washed four times in a simple cacodylate buffer (0.1 M, pH 6.8). Postfixation was conducted with 1% osmium tetroxide in the same cacodylate buffer at a 1 : 1 ratio for 1 h, at room temperature, and in the dark. After postfixation, basidiomatal pieces were rinsed in the cacodylate buffer for 2–3 min and dehydrated in a series of graded acetone solutions (30–100%), for 15 min in each concentration. All basidiomata were dried at critical point, mounted on stubs and sputter coated with gold palladium alloy (model S150B Sputter Coater, Edward, West Sussex, UK). All basidiomata were examined with a Jeol JSM 840-AE (Japan) scanning electron microscope, operated at 10 kV.

Molecular techniques.—Five mycelial disks were transferred from colonies actively growing for 10–15 d on PDA medium and inoculated into 50 mL of PD (potato dextrose) broth in 150 mL Erlenmeyer flasks and incubated at 25 ± 2 C for 10 d, with a photoperiod of 12 h and constant agitation at 120 rpm. Mycelial mats for DNA isolation were harvested by washing, and subsequently lyophilized and stored at −20 C. DNA was extracted according to Raeder and Broda (1985). DNA quantification and quality was estimated by comparison on 0.8% agarose gels with the high DNA Mass Ladder (50–5 ng/μL) (Gibco, São Paulo, Brazil). DNA concentrations were adjusted to 25 ng/μL.

The genomic sequence of nuclear ribosomal DNA (nrDNA), including the internal transcribed spacers (ITS) 1 and 2 and the 5.8S RNA gene, was amplified by PCR (polymerase chain reaction) using the universal primer pair

TABLE I. Isolates of *Crinipellis* and other *Tricholomataceae* used in the molecular studies

Isolates Accession No.	Origin (City/ State/Country)	Host species	Original material	GenBank Accession No. ITS1-5.8S-ITS2 rDNA
CANG/1*	Canavieiras/BA/Brazil	*Theobroma cacao*	Basidioma in culture	AY317131 *C. perniciosa*
UB (Mycol. Col.) 1998 (IL 9/8)*	Ilhéus/BA/Brazil	*Theobroma cacao*	Basidioma in culture	AY317132 *C. perniciosa*
BE12/18*	Belém/PABrazil	*Theobroma cacao*	Basidioma in culture	AY317133 *C. perniciosa*
UB (Mycol. Col.) 2021 (MA 5/5)*	Manaus/AM/Brazil	*Theobroma cacao*	Basidioma in culture	AY317134 *C. perniciosa*
UB (Mycol. Col.) 2027 (RO 8/8)*	Ouro Preto/RO/Brazil	*Theobroma cacao*	Basidioma in culture	AY317135 *C. perniciosa*
UB (Mycol. Col.) 2041 (LO 11/9)*	Lavras/MG/Brazil	*Solanum lycocarpum*	Basidiome/necrotic brown broom	AY317136 *C. perniciosa*
UB (Mycol. Col.) 2053 (CP 2/2)*	Itumirim/MG/Brazil	*Heteropterys acutifolia*	Basidiome/necrotic brown broom	AY317137 *C. brasiliensis* sp. nov.
UB (Mycol. Col.) 2054	Manaus/AM/Brazil	*Theobroma grandiflorum*	Basidiome/necrotic brown broom	AY317127 *C. perniciosa*
UB (Mycol. Col.) 2055	Planaltina/DF/Brazil	*Heteropterys nervosa*	Basidiome/necrotic brown broom	AY317126 *C. perniciosa*
FA617	Itajuípe/BA/Brazil	*Theobroma grandiflorum*	Basidiome/necrotic fruit tissue	AY317129 *C. perniciosa*
FA619	na/Bahia/Brazil	*Solanum paniculatum*	Basidiome/necrotic brown broom	AY317130 *C. perniciosa*
na	na/na/Brazil	*Theobroma grandiflorum*	vegetative dikaryotic mycelium	AY216468[G] *C. perniciosa*
na	na/na/Brazil	*Theobroma subincanum*	vegetative dikaryotic mycelium	AY216469[G] *C. perniciosa*
CP-37 (CEPLAC)	na/Bahia/Brazil	*Solanum paniculatum*	vegetative dikaryotic mycelium	AY216470[G *C. perniciosa*
na	na/na/Brazil	*Solanum paniculatum*	vegetative dikaryotic mycelium	AY176316[G] *C. perniciosa*
CP-83 (CEPLAC)	na/Bahia/Brazil	*Herrania sp.*	vegetative dikaryotic mycelium	AY21647[G] *C. perniciosa*
CP-104 (CEPLAC)	na/Bahia/Brazil	*Capsicum frutescens*	vegetative dikaryotic mycelium	AY216472[G] *C. perniciosa*
RNBP1	Quillabamba/Peru	*Theobroma cacao*	vegetative dikaryotic mycelium	AF335590[G] *C. perniciosa*
na	na	na	na	AY230255[G] *Crinipellis roreri* var. *gileri*
strain03	na/France (1983)	na	na	U54816[G] *Armillaria ostoyae*
strainM1	na/France (1969)	na	na	U54817[G] *Armillaria mellea*
strainCBB-361	na	na	vegetative dikaryotic mycelium	AY216476[G] *Marasmius* sp.
strain97	na/Thailand	na	na	AY244623[G] *Termitomyces* sp.

AM=Amazonas state; BA=Bahia state; MG=Minas Gerais state; PA=Pará state and RO=Rondônia state, DF=Distrito Federal.
UB (Mycol. Col.) = Mycological Collection of Reference, University of Brasília.
FA = Fazenda Almirante (Cocoa Research Center).
CEPLAC = Comissão Executiva do Plano da Lavoura Cacaueira.
[G] = data obtained from GenBank.
na = not available.
* Isolates studied in Arruda et al (2003a, 2003b).

ITS1 and ITS4 (White et al 1990). Eleven putative isolates of *C. perniciosa* representing seven plant hosts and 11 geographical origins were sequenced (TABLE I). Each 25 µL reaction volume contained 25 ng of DNA, 0.2 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 50 pmol of each primer, 2.5 U of Taq DNA polymerase and two drops of mineral oil. The amplification program comprised 35 cycles of 95 C for 1 min, 55 C for 1 min and 72 C for 1.5 min, and an additional extension step of 72 C for 10 min. All reactions

TABLE II. Morphometric characteristics of *Crinipellis* collections associated with four different host species belonging in three distinct botanical families

Characteristics	Host Plants/Family			
	Heteropterys nervosa *Malpighiaceae*	*Solanum lycocarpum* *Solanaceae*	*Heteropterys acutifolia* *Malpighiaceae*	*Theobroma cacao* *Sterculiaceae*
N° of basidiomata in LM/SEM analyses	05/03	05/03	08/03	05/30
Locality [Accession N°]	Planaltina/DF/Brazil [UB (Mycol. Col.) 19193]	Lavras/MG/Brazil [UB (Mycol. Col.) 19197]	Itumirim/MG/Brazil [UB (Mycol. Col.) 19198]	BA/Brazil: Santo Amaro [UB (Mycol. Col.) 19187], Ilhéus [UB (Mycol. Col.) 19188], Canavieiras [UB (Mycol. Col.) 19189, 19199] and Ipiaú [UB (Mycol. Col.) 19190], Ouro Preto/RO/Brazil [UB (Mycol. Col.) 19191/19199]; Belém/PA/Brazil [UB (Mycol. Col.) 19194]; Manaus/AM/Brazil [UB (Mycol. Col.) 19196]
Morphology of cheilocystidia	Sub-cylindrical, lageniform to obclavate	Sub-cylindrical, lageniform to obclavate	Obpiriform, lageniform to mucronate	Sub-cylindrical, lageniform to obclavate
Basidia (average) μm	23–31(27) × 5–8(6.5)	25–34(29) × 5–8(6.5)	30–39(34.5) × 4–8(6)	26–31(28) × 4–8(6)
Basidiospores (average) μm	Ellipsoid 9–12(10.5) × 4–7(5.5)	Ellipsoid 9–13(11.5) × 5–7(6)	Ellipsoid 10–14(12) × 5–7(6.5)	Ellipsoid 8–12(10) × 5–7(6.5)
Cheilocystidium (average) μm	26–41(32) × 9.7–12(11)	35–51(42) × 9.7–11(10)	28–37(31.4) × 10–16(12)	39–49(43) × 12–14(13)
Trichodermial hairs diam (average) μm	7.4–12.3(9.0)	7.4–12.3(9.5)	3.7–4.9(4.6)	7.4–12.3(9.6)
Pileus color and diam (mm)	Uniformly light pink 16.6–20.5(18.7)	Uniformly deep crimson red 7.4–14.2(10.7)	Uniformly light pink 8–9.2(8.6)	Crimson red fading to light pink 7.8–11.9(9.1)
Tukey*† test (basidia dimension)	161.10 b	196.30 a	202.82 a	176.66 b
Tukey*† test (basidiospore dimension)	55.30 c	72.46 b	81.44 a	55.26 c

AM = Amazonas state; BA = Bahia state; MG = Minas Gerais state; PA = Pará state and RO = Rondônia state, DF = Distrito Federal.
* Values within a row followed by the same letter do not differ according to Tukey's test ($P \cong 0.05$).
† Tukey test performed with morphometric analysis data (length and width) of 50 basidia and 50 basidiospores.

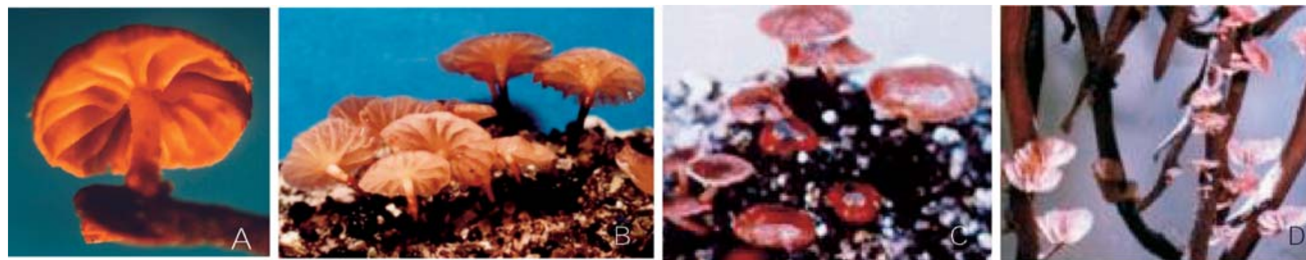


FIG. 1. Macroscopic differences among the four types of basidiomata of *Crinipellis* isolates. A. Basidiome from *H. acutifolia* UB (Mycol. Col.) 19198. B. Basidiomata from *T. cacao* UB (Mycol. Col.) 19192. C. Basidiomata from *S. lycocarpum* UB (Mycol. Col.) 19197. D. Basidiomata from *H. nervosa* UB (Mycol. Col.) 19193.

were carried out in a Stratagene Robocycler 96 thermocycler. PCR products were visualized by UV examination of ethidium bromide (0.5 μg/mL in water) stained 1.5% agarose gels and photographed with the Eagle Eye II photodocumentation system (Stratagene). To estimate DNA band sizes *Bam*HI, *Eco*RI and *Hin*dIII-digested phage λ (Gibco, São Paulo, Brazil) was included as a size marker on all gels.

Nuclear rDNA ITS PCR products were screened for restriction sites using a representative set of isolates of *Crinipellis*. Restriction digestion was conducted with 5 μL of PCR product and 5–10 units of these selected restriction enzymes: *Hinf* I, *Hin*PI, and *Msp*I. Restriction fragments were separated on 3% agarose gels and visualized and documented as previously described. Fragment sizes were calculated by comparison with a 100 bp ladder size marker (Gibco, São Paulo, Brazil). For each restriction endonuclease, digested PCR product fingerprints were compared in all isolates of *Crinipellis*. PCR products were purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, New Jersey) with final DNA concentrations adjusted to 100–200 ng/μL. PCR products were sequenced in both directions with a MegaBace 1000 capillary sequencer (Amersham Biosciences, Piscataway, New Jersey). Sequencing reactions comprised 1 μL of ITS PCR product (100 ng/μL), 4 μL of DYEnamic ET DYE Terminator Cycle Sequencing Kit for MegaBace (Pharmacia Biotech, Denver, Colorado), 0.5 μL of primer (0.5 μM) and Milli-Q water to a final volume of 10 μL. PCR conditions were: an initial step of 96 C for 2 min, 35 cycles of: 96 C for 10 s, 50 C for 5 s and 60 C for 4 min. Primers ITS1 and ITS4 were used individually in the sequence reaction. Sequences of a 611 bp length, generated from 11 isolates were deposited in GenBank, with these accession numbers: AY317126, AY317127 and AY317129–AY317137 (TABLE I).

Phylogenetic analysis.—Single spore cultures of basidiomata used in the morphological studies were the same used for the sequence analyses. A dataset was constructed of 23 ITS rDNA sequences, including 11 sequences of *Crinipellis* isolates generated in this study. For comparative purposes seven sequences of *C. perniciosa* and five sequences of other genera and species within the *Tricholomataceae* (outgroup) were downloaded from Genbank (TABLE I). Sequences were aligned with Clustal W (Thompson et al 1994). Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 2 (Kumar et al 2001). Parsimony analyses were performed with the close-neighbor-interchange method (CNI) with search in level 3 and a random addition tree with 100 replications. All characters were unordered and equally weighted and gaps were treated as missing data, with all positions included. A neighbor-joining (NJ) analysis (Saitou and Nei 1987) using the Kimura-2-parameter as distance option was used to derive a distance tree. Confidence intervals were determined in both parsimony and distance analyses, via bootstrap analysis using one thousand replicates. Alignments are available at TreeBASE (www.treebase.org).

RESULTS

Morphology.—Sixty-two basidiomata from four different host species were examined in macroscopic observations, 23 were studied by LM, and 39 using SEM (FIGS. 1–3). The *Crinipellis* isolates from *H. acutifolia* morphologically were distinct from all other isolates studied (FIGS. 1–3).

The basidiomata of the isolates from *T. cacao*, regardless of geographic origin, were found to be morphologically similar with plane pilei with straight margins, gill spacing distant or subdistant, with eroded margins and not attached to the stipe. However the isolates produced basidiomata with pilei varying from light pink to crimson red (FIG. 1B). Isolates of *C. perniciosa* from *T. cacao* (UB [Mycol. Col.] 19190) (TABLE II) and those from *S. lycocarpum* presented deep crimson red pilei. Basidiomata from *S. lycocarpum* also were pigmented more uniformly (FIG. 1C) and occasionally were larger than observed in isolates from other hosts (data not shown). All isolates from *H. acutifolia*and isolates from *T. cacao* (not including UB [Mycol. Col.] 19190) showed basidiomata uniformly lighter in color, with flat-umbonate pilei (FIG. 1A). Finally, *Crinipellis* basidiomata from *H. nervosa* were light pink in color, presenting larger pilei diameter (TABLE II, FIG. 1D).

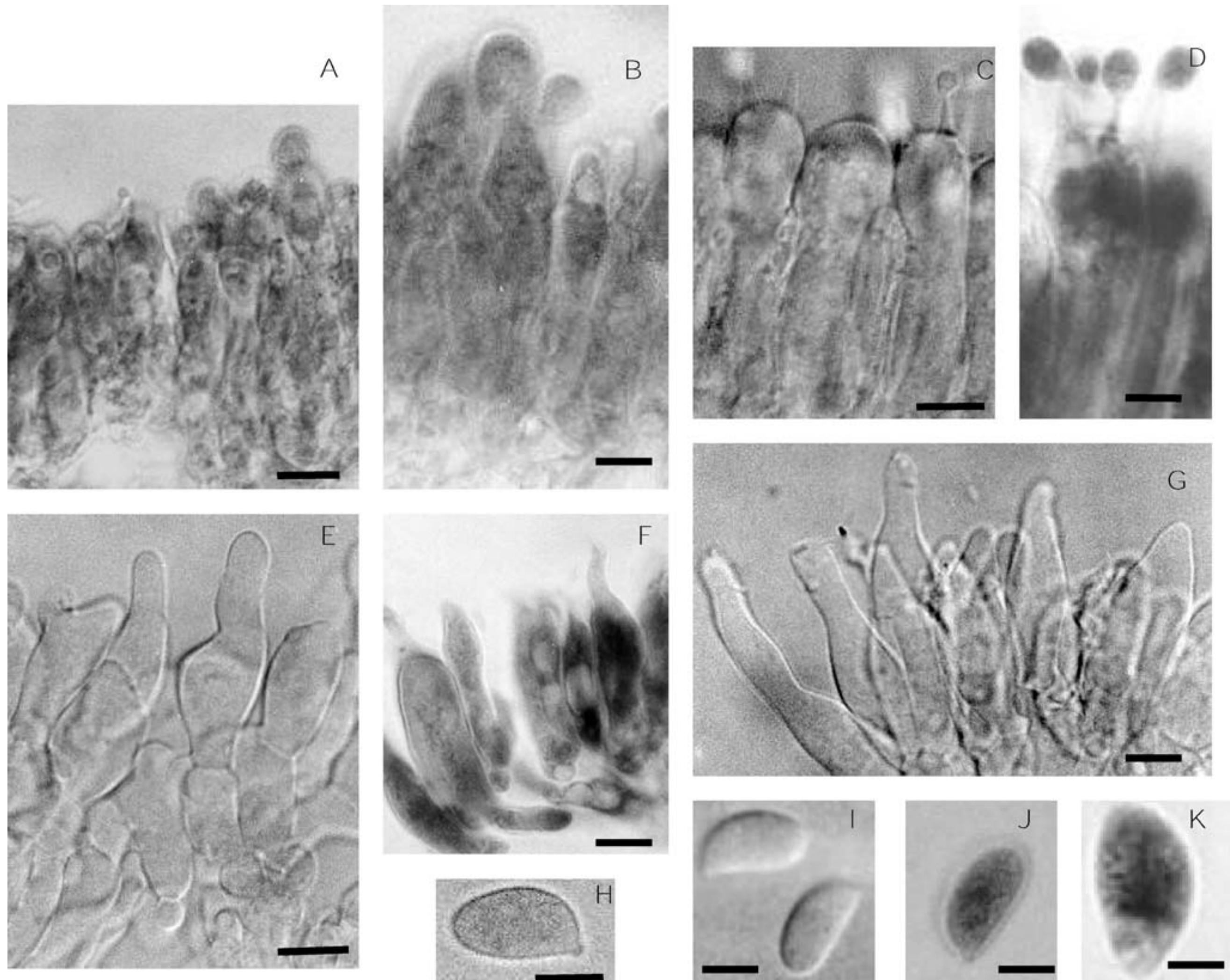


FIG. 2. Morphology of *Crinipellis* isolates from *H. acutifolia* ([UB [Mycol. Col.] 191980 (A, B, E, F, H), *T. cacao* UB (Mycol. Col.) 19199 (C, D, I) and *S. lycocarpum* UB (Mycol. Col.) 19197 (G, J, K). A. Hymenium with basidia and one basidiospore. B. Hymenium with basidia and two basidiospores. C. Hymenium with basidia and basidiole. D. Basidium with four basidiospores attached. E and F. Cheilocystidia obpiriform, lageniform to mucronate. G. Cheilocystidia subcylindrical, lageniform to obclavate. H. Basidiospore with visible apiculum. I.J. and K. Basidiospores. Bars: 2B–2D, 2F, 2G–2K = 5 μm; 2A and 2E = 10 μm).

A pure white spore print was a conserved character within the genus *Crinipellis*. Similar morphological characteristics observed in isolates from *H. acutifolia* and other species within the genus, not including dimension comparisons, also comprised ellipsoid, hyaline, smooth, apiculate inamyloid spores (FIG. 2H). Hyphae with clamp connections, characteristic of dikaryotic mycelia, also were common for all isolates examined. Hyphal trama of pilei and stipes did not show noticeable differences among isolates studied. Basidial ontogeny was identical for all isolates from *H. acutifolia*, *T. cacao*, *S. lycocarpum* and *H. nervosa*.

The presence of pseudoamyloid hairs in pileus is a common feature of the *Crinipellis* genus, with all specimens studied showing an identical trichoderm consisting of numerous thick-walled hairs. The pileal hairs were concentrated in the central portion of the pileus, which showed ornamented margin covered with long hairs. These hairs were white or red when fresh becoming hyaline when dried or in old basidiomata. In the specimen found on *H. acutifolia* the pileal hairs were cylindrical and characteristically thinner (17–110 × 4–5 μm) than those from other hosts (TABLE II, FIG. 3H).

Basidial morphology that frequently showed four basidiospores, and cheilocystidial morphology that showed subcylindrical, lageniform to obclavate shape (regularly ''bottle-shaped''), were similar among all *Crinipellis* isolates, except in those from

H. acutifolia that showed 1–3- instead of 4-spored basidia (FIG. 3A, B, D), with short obpyriform, lageniform to mucronate cheilocystidial shape (FIG. 2E, F, 3I) and also several instances of direct basidial germination (FIG. 3G) giving rise to a simple hypha. In addition basidia and basidiospores produced in *H. acutifolia* and *S. lycocarpum* were significantly larger than those from other hosts (Tukey test, $P \cong 0.05$) (TABLE II).

Molecular data.—PCR-based amplification of the ITS1, ITS2 regions and 5.8 S rDNA gene yielded a product of approximately 750 bp in all isolates examined. Amplification was reproducible both at higher and lower primer annealing temperatures. Restriction digestions of the ITS region produced identical profiles in all isolates. All restriction sites of the three endonucleases were identified.

Alignment of the DNA sequence data within the ITS1, ITS2 regions and 5.8S rDNA gene revealed variability among the different species studied. The complete alignment included 847 positions, with 265 conserved, 487 variable and 331 parsimony-informative positions. Parsimony analysis of the rDNA region resulted in 98 equally parsimonious trees, with a single most parsimonious tree recovered (tree length 490, with CI = 0.8693, RI = 0.8320, RCI = 0.7233), with six principal clades (FIG. 4). Isolates representing *C. perniciosa* were grouped into one major clade. This clade could be divided into three groups: (i) *C. perniciosa* isolates from *T. cacao*, *T. grandiflorum*, *T. subincanum*, *Herrania* sp., *H. nervosa*, *S. paniculatum* and *Capsicum frutescens* hosts; (ii) *C. perniciosa* isolates from *S. lycocarpum* (UB [Mycol. Col.] 2041); and (iii) *C. perniciosa* from *S. paniculatum*, that subdivided into subgroups (CP-37-AY216470 and FA619). The *Crinipellis* isolate from *H. acutifolia* (UB [Mycol. Col.] 2053) formed a single clade. Bootstrap analysis showed that the branch point separating the *C. perniciosa* major clade from the *Crinipellis* isolate from *H. acutifolia* had a confidence interval of 60%. The isolate of *C. roreri* (AY230255) formed a separate clade, supported with a bootstrap of 99%. The four remaining species *Marasmius* sp. (100% bootstrap), *Armillaria* spp. (U54816, U54817) and *Marasmiellus stenophyllus* were maintained in a single clade each. The *Armillaria* spp. clade (99% bootstrap) was divided in two groups.

Within the major clade there was no observable distinction based on host or geographical origin. Data from nrDNA sequences revealed that some S-biotype isolates (*Capsicum frutescens*, *S. paniculatum* and *S. lycocarpum*) and the H-biotype isolates (*H. nervosa*) were grouped indistinctly with C-biotype isolates (*T. cacao*, *T. grandiflorum*, *T. subincanum* and *Herrania* spp.). Distance analysis confirmed that the isolate from *H. acutifolia* (UB [Mycol. Col.] 2053) was distinct from all other isolates of *C. perniciosa*, at a distance level of ˜0·015 (FIG. 5), suggests that this is a unique species.

TAXONOMY

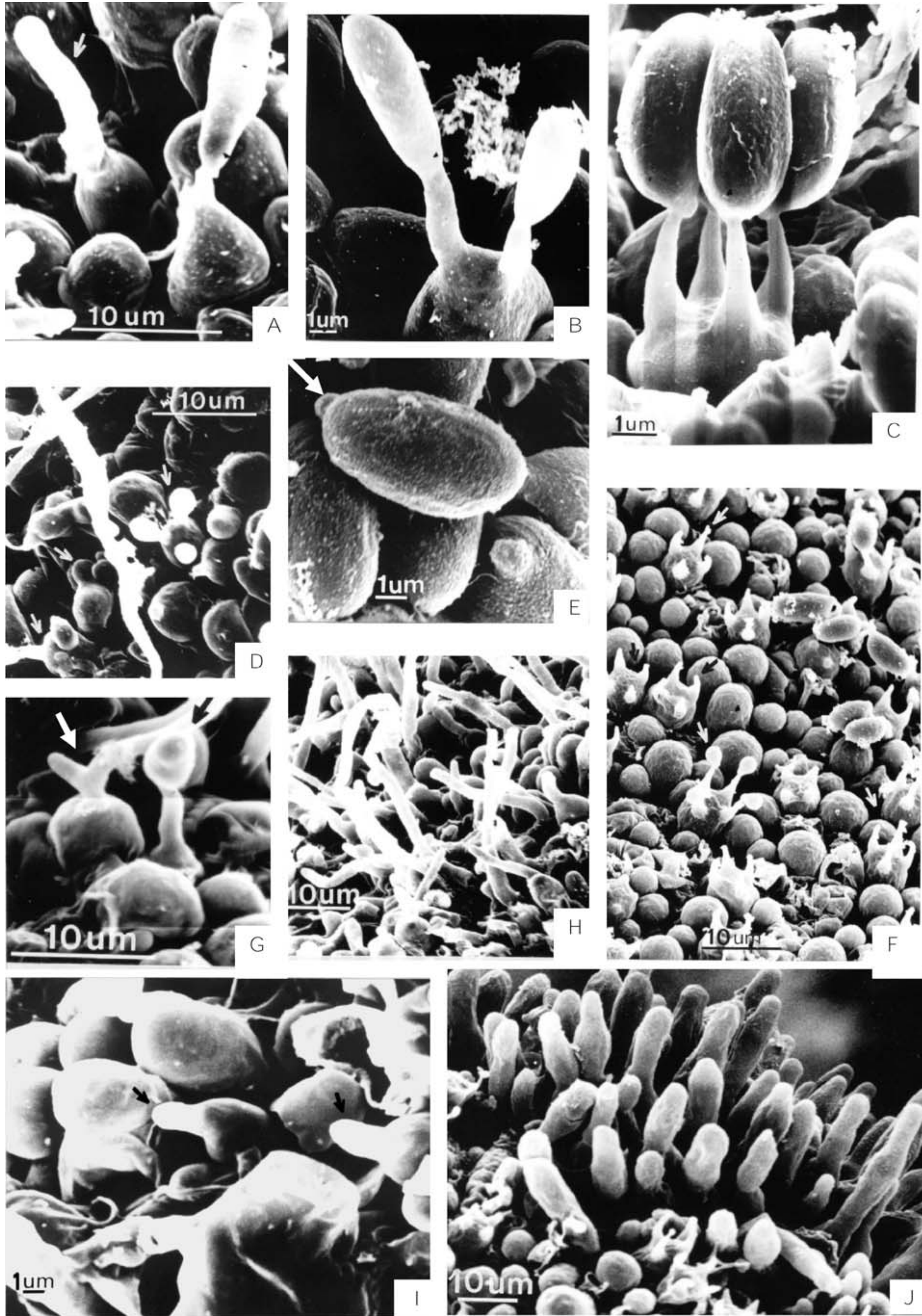
Crinipellis isolates from *Theobroma cacao*, *Solanum lycocarpum*, *Heteropterys nervosa* and *H. acutifolia* can be morphologically compared (TABLE II). Color of the basidiomata, cheilocystidial shape and size, basidium and basidiospore size and the dimensions of the trichodermal components show clear differences among specimens (TABLE II), indicating that the specimen from *H. acutifolia* does not belong in *Crinipellis perniciosa*. Thus the *Crinipellis* specimen from *H. acutifolia* will be described here and allocated in a new species of the genus.

Crinipellis brasiliensis M.C.C. de Arruda, G. Sepúlveda, R.N.G. Miller, M.A.S.V. Ferreira & M.S.S. Felipe, sp. nov. FIGS. 1–3

Pilei carnosi, convexo-campanulati, sulcinervii, venatio radiata ex centro palido roseo vel roseolo et depresso oriunda, albidescentes versus margines tenues curvatos, trichodermici, stipitati, 8–9.2 mm diam. Pili trichodermici paginae pileorum insidentes, zonis concentricis formantes, palido brunnei vel brunneo rosi, simplices, parietibus crassis, cylindrici, apicibus rotundatis, 17–110 × 4–5 μm. Lamellae 120–250 μm latae, palido brunneae in pileis maturis, albae in speciminibus exsiccates. Stipite excentrici, solidi, erecti vel curvati, albi, base sub-bulbosa et rufa. Basidia clavate, 1–3 basidiosporis, 30–39 × 4–8 μm. Massa sporae alba. Basidiosporae ellipticae, hyalinae, laeviae, apiculatae, non amyloideae, 10–14 × 5–7 μm. Cheilocystidia lageniformia, sparsa, 28–37 × 10–16 μm.

Pilei fleshy, campanulate, with a depressed rose to pink center giving rise to radiating sunken veins, whitish toward the thinner curved borders, trichodermatous, stipitate, 8–9.2 mm wide, Trichodermal hairs forming concentric rings on the surface of the pilei, light brown to brownish rose, simple, thick-walled, cylindrical with rounded tips, 17–110 × 4–5 μm. Lamellae 120–250 μm wide, light brown thin in mature pilei, white in dried pilei. Stipes eccentric, solid, erect or curved, white, with a reddish subbulbous base. Basidia clavate, 1–3-spored, 30–39 × 4–8 μm. Spore print white. Basidiospores ellipsoid, hyaline, smooth, non-amyloidal, 10–14 × 5–7 μm (FIGS. 1B, C, 2G). Cheilocystidia lageniform with a thin apex, sparse, 28–37 × 10–16 μm (FIGS. 1B, C, 2G).

Specimens examined. BRAZIL. MINAS GERAIS: Itumirim. On dry fan brooms of *Heteropterys acutifolia* Adr. Juss., 19 Oct 1999, *Maricília C C de Arruda 43* (*HOLOTYPE* UB [Mycol. Col.] 19198); DISTRITO FEDERAL: Planaltina, ''Cer-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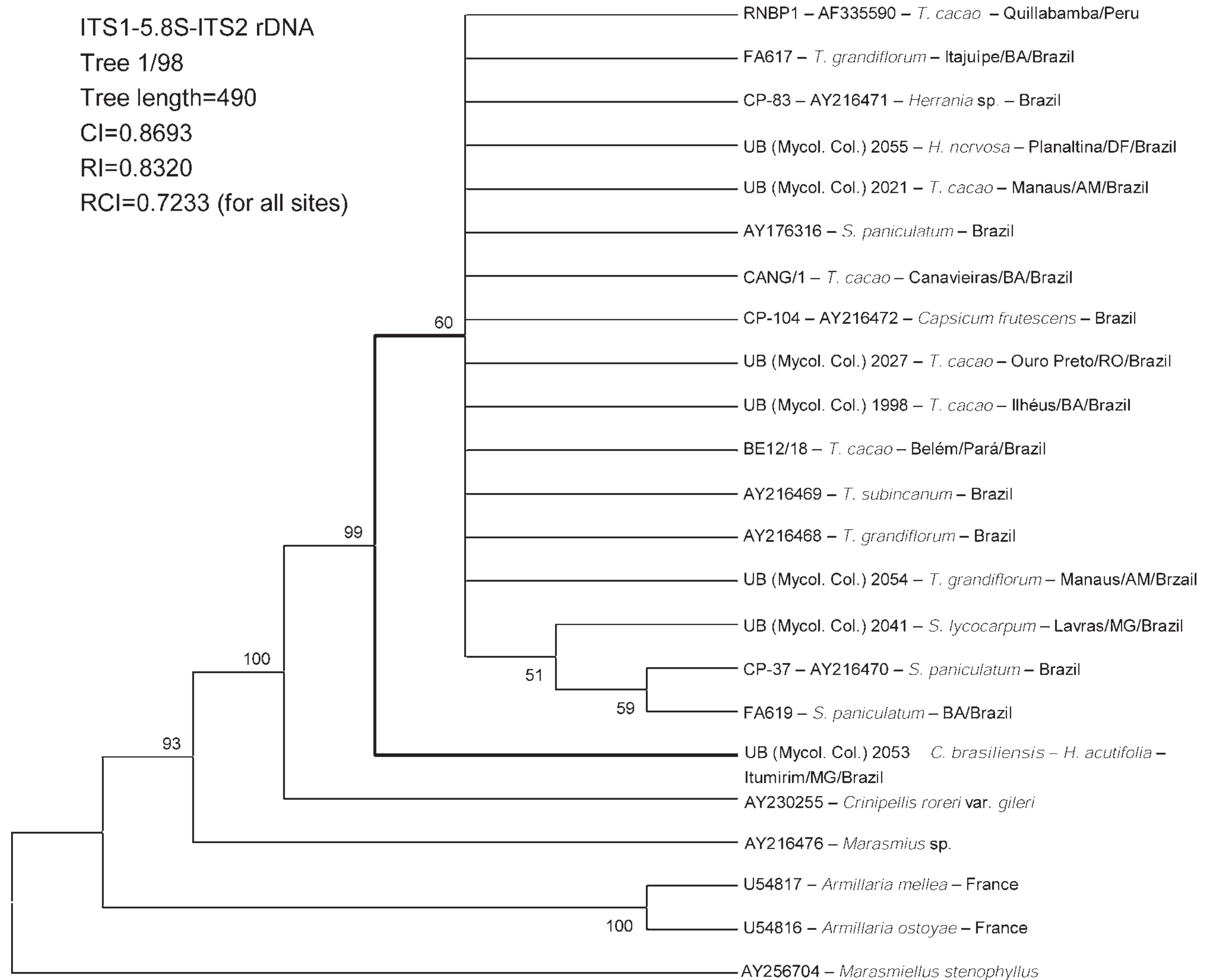

FIG. 4. Phylogram based on DNA sequence data from ITS1, ITS2 and the 5.8S RNA gene generated for 11 *Crinipellis* isolates, compared with sequences from seven isolates of *C. perniciosa*, one isolate of *C. roreri* and four isolates from different genera of the *Tricholomataceae* (GenBank). One of 98 equally parsimonious trees (490 steps, CI = 0.8683, RI = 0.8320). Bootstrap values greater than 50% are indicated along nodes.

radão'' reserve at Embrapa-Cerrados. On dry fan brooms of *Heteropterys nervosa* Adr. Juss, 16 Jul 2002, *Maricília C C de Arruda 37* UB (Mycol. Col.) 19193; MINAS GERAIS: Lavras. On dry fan brooms of *Solanum lycocarpum* St Hil., 19 Oct 1999, *Maricília C C de Arruda 39* UB (Mycol. Col.) 19197; BAHIA: Canavieiras. From culture of the fungus isolated from *Theobroma cacao* L., 16 Jun 1999, *Maricília C C de Arruda 45* UB (Mycol. Col.) 19199.

DISCUSSION

The results of LM and SEM observations clearly indicated that the isolates of *C. brasiliensis* from *H. acutifolia* are distinct from *C. perniciosa* from *T. cacao, S. lycocarpum* and *H. nervosa*. In addition the molecular data fully support this indication. Morphological comparison using LM revealed variability

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FIG. 3. Ultrastructure of basidia of *Crinipellis* isolates from *H. acutifolia* UB (Mycol. Col.) 19198 (A, B, D, G, H, I) and *T. cacao* UB (Mycol. Col.) 19199 (C, E, F, J). A. Basidia with one basidiospore. B. Basidium with two basidiospores at different stages of maturity, cylindrical and elongated. C. Basidium globose with four sterigmata and four basidiospores, at same maturity stage. D. Overview of basidia with one and three basidiospores (arrows). E. Slightly rough ellipsoid basidiospores with distinct apiculum (arrow). F. Overview of basidia with four basidiospores (arrows). G. Basidium with direct germination (white arrow) and basidium with one basidiospore (white arrow). H. Pileal hairs in the center portion of the pileus. I. Cheilocystidia thin, elongated and irregular (arrows). J. Cheilocystidia sub-cylindrical, lageniform to obclavate.

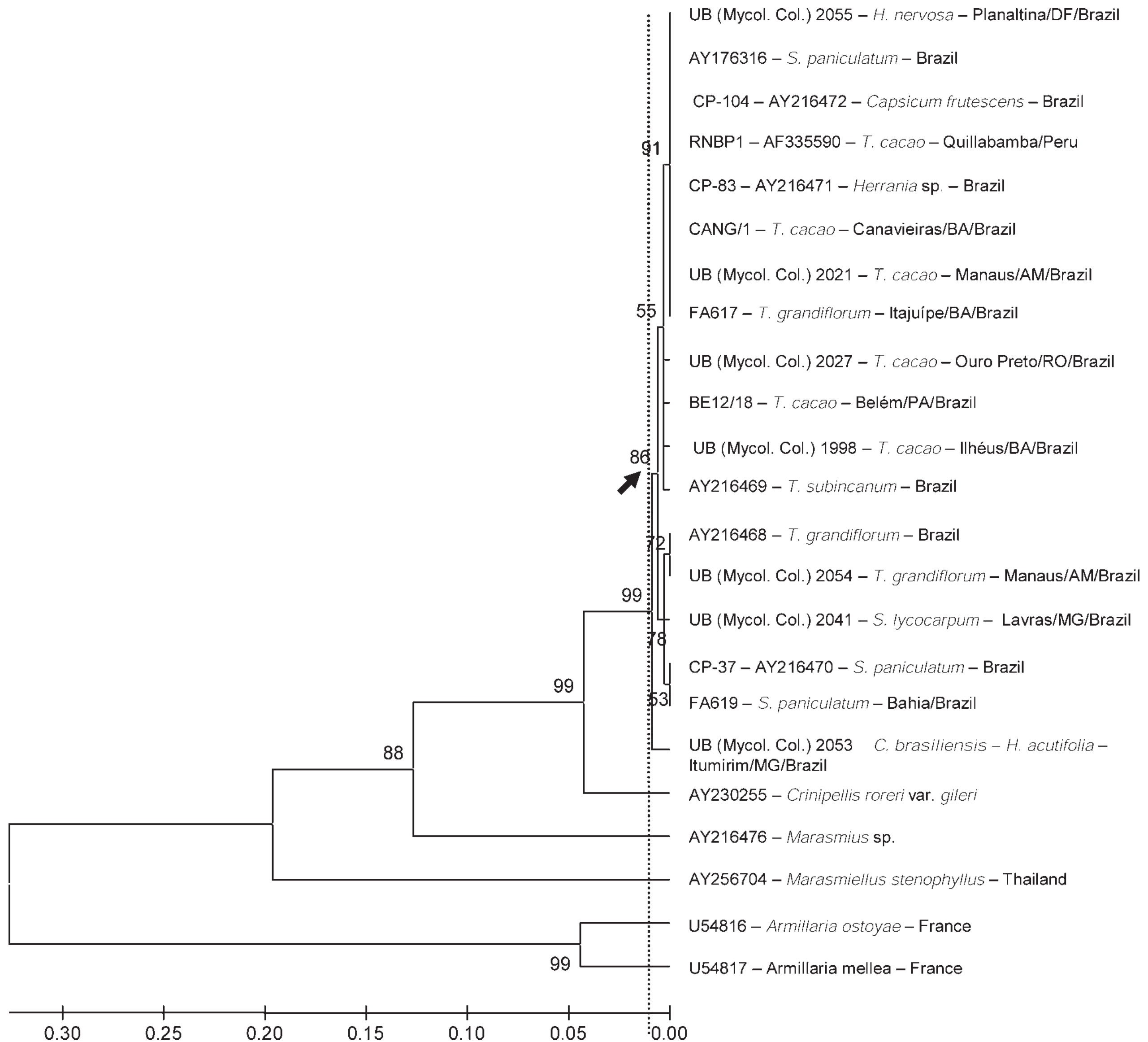

FIG. 5. Dendrogram based on DNA sequence data from ITS1, ITS2 and the 5.8S RNA gene generated for 11 *Crinipellis* isolates, compared with sequences from seven isolates of *C. perniciosa*, one isolate of *C. roreri* and four isolates from different genera of the *Tricholomataceae* (GenBank), using neighbor-joining (NJ) cluster analysis. The scale represents genetic distance obtained using Kimura 2-parameter distance. *C. brasiliensis* is separated from the other of *C. perniciosa* isolates at a distance level of ˜ 0·015 (dotted line and black arrow). Bootstrap values greater than 50% (based on 1000 bootstrap replicates) are indicated at each branch point.

among isolates of *C. brasiliensis* in terms of morphometric characteristics of the basidia and basidiospores, (FIG. 3A, B, D), trichodermal dimensions (TABLE II), cheilocystidia length and shape (FIGS. 2E, F, 3I), and basidial length. Isolates from *H. acutifolia* also showed morphologically distinct trichodermal hairs on the pileus (FIG. 3H).

SEM observations also revealed that isolates from *H. acutifolia* differ from the others in terms of shape of dikaryotic hyphae and cheilocystidia, shape and size of the basidiospores and number of basidiospores per basidia. Basidiospores in isolates from *H. acutifolia* showed different sizes in the same basidium, indicating a sequential process of sporulation (FIG. 3B, D).

Viana Júnior (2001) observed that *Crinipellis* isolates from *H. acutifolia* and *S. lycocarpum* genetically were distinct from those of *T. grandiflorum*, *T. cacao*, *T. bicolor* and *Herrania* sp., on the basis of somatic incompatibility. Hedger et al (1987) also found differences in basidiomatal size and shape of cheilocystidia among isolates from *T. cacao* and from *A. verrucosa*. In other studies differentiation in S- and L-biotype isolates also has been reported by Griffith

and Hedger (1994), revealing high frequency (ca. 7%) of binucleate basidiospores. In contrast to our findings based upon morphological and molecular analyses Resende et al (2000) did not find morphological differences between isolates of *Crinipellis* from *T. cacao* and from *H. acutifolia*. These authors reported that in isolates of *Crinipellis* from *H. acutifolia* the basidiospores were hyaline, ellipsoid and reniform with dimensions 6.2–9 × 3–5 μm, showing a tomentum on the surface of the pileus and stipe, with hair tips swollen or rhomboid and rounded, which together with morphological data based on cheilocystidia, basidioles and basidia with four sterigmata indicated that those characteristics were consistent with those described by Singer (1942, 1976) for *C. perniciosa*. On the other hand the present study showed considerable differences between isolates of *Crinipellis* from *H. acutifolia* and those from three other hosts including *T. cacao*, which were considered sufficient to establish a new taxon within *Crinipellis*. Thus it is possible that the broom material studied by Resende et al (2000) was infected by more than one *Crinipellis* species or simply infected with *C. perniciosa*.

The results based on morphological analysis were supported further and confirmed by molecular analyses of rDNA regions from 11 isolates of *Crinipellis* from different geographic origins and hosts (TABLE I). Size comparisons of PCR-amplified rDNA regions and RFLP analysis did not reveal any polymorphisms. Given that this study used only a limited number of restriction enzymes, it is possible that limited intraspecific differences were not detectable by RFLP methods. However alignment of DNA sequence data for this region revealed variability among all the isolates studied. Phylogenetic analysis of the dataset showed that the isolate of *C. brasiliensis* from *H. acutifolia* UB (Mycol. Col.) 2053 is separated from all other isolates of *C. perniciosa* (FIG. 4). This suggested that the isolate from *H. acutifolia* was not supported as conspecific, within the major clade of *C. perniciosa*. The dendrogram derived from distance tree analysis also showed that the isolate from *H. acutifolia* is distinct from the *C. perniciosa* group (FIG. 5).

In contrast to earlier studies using RAPD, where molecular variability in *C. perniciosa* was linked with geographical origin (Andebrhan and Furtek 1994, Andebrhan et al 1999, Gomes et al 2000, Niella et al 2000), this study reveals no such correlation. Isolates from different states and countries (Brazil and Peru) often grouped within a single clade. This possibly might be attributed to the greater conservation of the rDNA ITS regions when compared with RAPD markers. Such conservation has played a major role in increasing the understanding of the relationships within the *Basidiomycota*. Considered a useful marker, the rDNA ITS region has been used to determine the phylogenetic relationships among *Armillaria* spp. (Coetzee et al 2001), to show phylogenetic differences between *Lentinula* species from the Asian-Australasian region (Nicholson et al 1997), to differentiate pathogenic fungi at the species level (Vilgalys and Gonzalez 1990), to investigate the genetic diversity of *Ganoderma* (Miller et al 1999), to examine the genetic divergence of *Pleurotopsis longinqua* from different geographical area (Hughes et al 1998) and for taxonomic studies of several genera, including *Marasmius* (Moncalvo et al 2002).

Arruda et al (2003a) used other molecular markers to determine variability among specimens of different hosts and geographical origins. Phenetic analyses of banding patterns by ERIC-PCR (enterobacterial repetitive intergenic consensus-based polymerase chain reaction) distinguished isolates on the basis of host with *T. cacao*-derived isolates only 20% similar to those from *H. acutifolia* and *S. lycocarpum*.

Genetic differences were previously (Arruda et al 2003b) detected by RFLP and sequence analysis of the nuclear rDNA IGS regions that discriminated isolates from *T. cacao, S. lycocarpum,* and *H. acutifolia* and showed considerable intraspecific variability within each host derived group. Phylogenetic analysis in this study showed that some S-biotype isolates (UB [Mycol. Col.] 2041, CP-37- AY216470 and FA619) were separated in two subgroups into one major clade of *C. perniciosa* isolates, perhaps establishing an intermediate group in this clade; on the other hand, the H-biotype from *H.* nervosa and others S-biotype isolates from different hosts were grouped together indistinctly with C-biotype isolates. It thus is possible that intraspecific differences, in *C. perniciosa* isolates were detectable through rDNA sequence analysis but not detected by RFLP methods.

Differentiation of *C. perniciosa* on the basis of host also has been reported in other recent studies. For example, in a small scale molecular-based comparison of isolates of *C. perniciosa* from several Brazilian states and the same three host species, RAPD-derived polymorphisms distinguished isolates from different states and hosts. Thus isolates from *T. cacao* were separated from those of *S. lycocarpum* and *H. acutifolia* (Niella et al 2000). Gramacho et al (2002) characterized isolates of *C. perniciosa* from numerous host species on the basis of RAPDs, rDNA analysis and cross inoculation tests. The authors also reported that isolates from *Stigmaphyllon blanchetii* C.E. Anderson (*Malpighiaceae*) and *Solanum rugosum* to be genetically distinct from those of *T. cacao*.

As mentioned, cross inoculation testing of isolates

of *Crinipellis* from *H. acutifolia* on *T. cacao* previously has shown inconsistent results, with isolates reported as pathogenic on *T. cacao* (Resende et al 2000) or not (Viana Júnior 2001). Such an approach, while potentially informative for differentiation of host specific species or intraspecific groups in *Crinipellis*, may simply reflect technical problems or even difference in methods used for inoculation and maintenance of the inoculated plants.

In summary *C. brasiliensis* isolates from *H. acutifolia* were similar morphologically to *C. perniciosa* isolates from *T. cacao, S. lycocarpum* and *H. nervosa* in terms of the presence of cheilocystidia but distinct in terms of sporogenesis, and morphometry of cheilocystidia, basidia and basidiospores. These morphological differences supported by sequence-based comparison of the rDNA ITS region and 5.8S gene, indicated that the isolates from *H. acutifolia* represent a new species within the genus *Crinipellis*, here designated as *C. brasiliensis*.

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