

## The causal agents of witches' broom and frosty pod rot of cacao (*Theobroma cacao*) form a new lineage of Marasmiaceae

M.C. Aime<sup>1, 2</sup>

USDA-Agricultural Research Service, Systematic Botany and Mycology Lab, 10300 Baltimore Ave., Beltsville, Maryland 20705

W. Phillips-Mora

Cacao Breeding Program, CATIE 7170, Turrialba, Costa Rica

**Abstract:** The two most devastating diseases of cacao (*Theobroma cacao*)—the source of chocolate—in tropical America are caused by the fungi *Crinipellis pernicioso* (witches' broom disease) and *Moniliophthora roreri* (frosty pod rot or moniliasis disease). Despite the agricultural, socio-economic and environmental impact of these fungi, most aspects of their life cycles are unknown, and the phylogenetic relationships of *M. roreri* have yet to be conclusively established. In this paper, extensive phylogenetic analyses of five nuclear gene regions (28S rDNA, 18S rDNA, ITS, RPB1, and EF1- $\alpha$ ) confirm that *C. pernicioso* and *M. roreri* are sister taxa that belong in the Marasmiaceae (euagarics). Furthermore, these taxa form part of a separate and distinct lineage within the family. This lineage includes the biotrophic fungi *Moniliophthora pernicioso* comb. nov. and *M. roreri*, as well as one undescribed endophytic species. The sister genera to *Moniliophthora* are *Marasmius*, *Crinipellis* and *Chaetocalathus*, which consist mainly of saprotrophic litter fungi.

**Key words:** anamorphic basidiomycetes, cacao pathogens, cocoa, fungal taxonomy, molecular systematics

### INTRODUCTION

The two most devastating diseases of cacao (*Theobroma cacao* L.) in tropical America are caused by the fungi *Crinipellis pernicioso* (Stahel) Singer (witches' broom disease) and *Moniliophthora roreri* H.C. Evans, Stalpers, Samson & Benny (frosty pod rot or moniliasis disease). *Crinipellis pernicioso* belongs to a large order of basidiomycetes, the euagarics, which

contains few known pathogens, while *M. roreri* is widely believed to be an anamorphic ascomycete (Kirk et al 2001). Both fungi are indigenous to tropical South America and attack species of *Theobroma* L. and the closely-related genus *Herrania* Goudot, in particular, *T. cacao*, the source of chocolate. Both are able to disrupt host hormonal balance, provoking hypertrophy and hyperplasia followed by tissue necrosis (Holliday 1980). Moreover, the intercellular mycelium of both fungi is typically swollen and convoluted in marked contrast to mycelium growing intracellularly or in standard culture (Evans 1981). Together these diseases have caused relatively recent devastation to major cacao-farming regions in South and Central America (Pereira 1999, Phillips-Mora 2003), and are responsible for the plummet in tropical American cocoa production. Despite the socio-economic and environmental impact of these fungi, aspects of their life cycles, including mode of reproduction, have yet to be resolved, and phylogenetic relationships remain uncertain.

Initial infection by *C. pernicioso* basidiospores occurs in actively growing cacao meristems, causing a characteristic disorganized proliferation of new shoots in the host that are termed "witches' brooms" (Isaac et al 1993) (FIG. 1a). Potential crops are lost when clusters of flowers produced on "cushions" on the main trunk and older branches are infected, thus producing seedless strawberry- or carrot-shaped fruits (Pereira 1999). Additionally, *C. pernicioso* attacks cacao pods in the early stages of development, penetrating the husk and destroying the seeds from which chocolate is derived (FIG. 1b). After death of the broom tissue, fructifications of small pink agarics occur, which Stahel (1915) identified as the causal agent of the disease and named *Marasmius pernicioso* Stahel. At that time the disease had been known for approximately 20 years in northern South America (Stahel 1915).

By the time Singer transferred the pathogen to the genus *Crinipellis* Pat. it had already caused the failure of the cocoa industry in Suriname and spread to Trinidad, West Indies and Ecuador (Singer 1942). By 1980 the pathogen had continued to spread into Colombia, Peru, and Venezuela (Holliday 1980). The cocoa production of Bahia, Brazil, once a major world producer and exporter of chocolate, suffered a 95%

Accepted for publication 17 Sep 2005.

<sup>1</sup>Corresponding author. E-mail: cathie@nt.ars-grin.gov

<sup>2</sup>Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

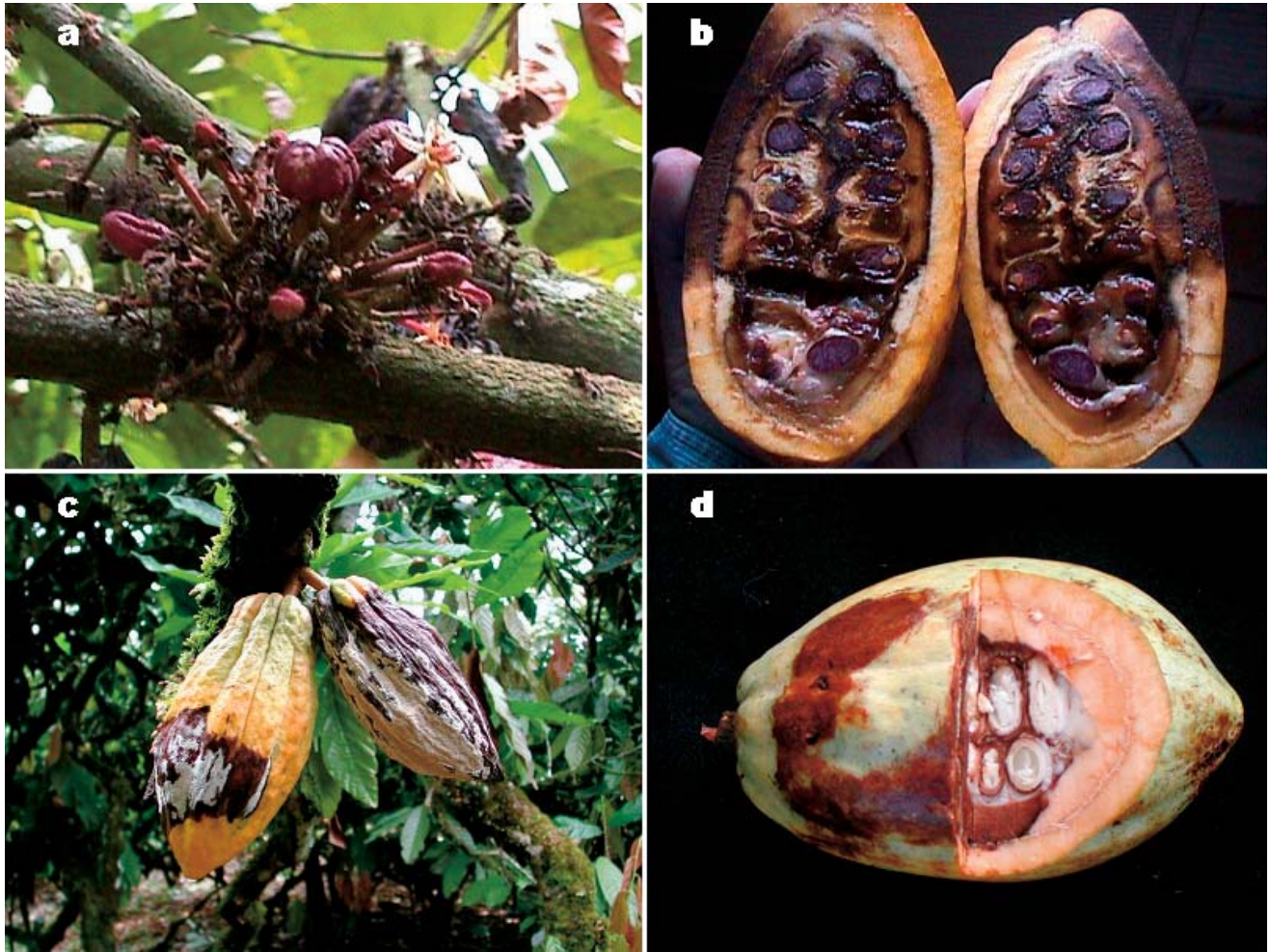


FIG. 1. Infection of the chocolate (*Theobroma cacao*) tree and pods by cacao pathogens *Moniliophthora* (*Crinipellis*) *perniciosa* and *Moniliophthora roreri*. a. Witches' broom of plant stems caused by *M. pernicioso* infection. b. Chocolate pods and seeds infected with *M. pernicioso*. c, d. Frosty pod rot caused by *M. roreri* on pods and seeds.

crop loss since the introduction of *C. pernicioso* in 1989 (Pereira et al 1990, Isaac et al 1993).

In contrast, *M. roreri* infection is limited to the fruits of *Theobroma* and *Herrania* species, causing internal and external pod damage that results in total loss of the pods (FIGS. 1c, d). The devastating effects and loss of cacao crops due to this pathogen have been dramatic. However, until the 1950s, frosty pod rot was confined to the northwestern part of South America (Colombia, Ecuador and western Venezuela) and therefore not of widespread interest to other cocoa-producing countries. However, its appearance in Panama in 1956 and Costa Rica in 1978 increased the geographic confines of the pathogen and changed perceptions of the disease (Evans 1986).

Evans et al (2003) consider that *M. roreri* is still in an invasive phase—having reached Nicaragua in 1980, Peru in 1988, Honduras in 1997, Guatemala in 2002, Belize in 2004, and Mexico in 2005 (Phillips-Mora

2003 and unpublished)—and is poised to devastate already crippled production in Bolivia and Brazil, once it arrives in those countries. Because this fungus has never been observed to form any type of fruiting body and the only propagules produced were considered to be conidia, the causal agent of frosty pod rot was originally described as an anamorphic ascomycete, *Monilia roreri* Cif. (Ciferri and Parodi 1933). Not until 1978 did a critical re-evaluation of *M. roreri* reveal the presence of dolipore septa (Evans et al 1978), a feature found only in basidiomycete hyphae. A new genus of anamorphic basidiomycete, *Moniliophthora* H.C. Evans, Stalpers, Samson & Benny, was erected to accommodate this taxon, whose placement within the basidiomycetes was unknown (Evans et al 1978). Recently, several studies have hypothesized that *M. roreri* might belong to the Tricholomataceae sensu lato (s.l.) (Phillips-Mora 2003, Phillips-Mora et al 2003), and might be closely

related to *C. pernicioso* (Evans 1981, Evans et al 2002, Phillips-Mora 2003, Griffith et al 2003).

The purpose of the present study was to determine the phylogenetic placement and relatives of *M. roreri*, and to evaluate the hypothesis that *M. roreri* and *C. pernicioso* are closely related. This was determined by phylogenetic analyses of DNA sequences from five different nuclear gene regions: large subunit ribosomal DNA (LSU); small subunit ribosomal DNA (SSU); internal transcribed spacer regions 1 & 2 and the intervening 5.8S ribosomal subunit (ITS); elongation factor 1- $\alpha$  (EF1- $\alpha$ ); and the largest subunit of RNA polymerase II (RPB1). As a result of the molecular study, a new combination, *Moniliophthora pernicioso* Aime & Phillips-Mora comb. nov. is proposed for the causal agent of witches' broom, and a complete taxonomic discussion of the cacao pathogens and related agarics is provided.

#### MATERIALS AND METHODS

*Specimens.*—Materials were obtained from a variety of sources, i.e. as fresh field collections, dried herbarium specimens, cultures or previously accessioned DNA sequences from GenBank (<http://www.ncbi.nlm.nih.gov/>). Three samples were acquired as extracted DNA from Manfred Binder (Clark University, Worcester, Massachusetts). The provenance of all materials is provided (TABLE I). Field collections were described in the fresh condition and then preserved by drying in silica gel in individual containers; prior to drying, a piece (approximately 1–4 mm<sup>3</sup>) of tissue was preserved in 500  $\mu$ L of 2 $\times$  CTAB (hexadecyltrimethylammonium bromide) extraction buffer (Hillis et al 1996) for later DNA extraction. Vouchers of all field collections used for DNA sequences have been deposited in the U.S. National Fungus Collections (BPI) in Beltsville, Maryland.

*DNA extraction.*—After receiving permission from the various herbaria, a small piece (1–2 mm<sup>3</sup>) of tissue was removed from dried collections and placed in 2 mL Bead Solution tubes of the UltraClean Plant DNA Isolation Kit and extracted per the manufacturer's instructions (MoBio Laboratories, Inc., Solana Beach, California), except tissue was left to soak overnight in the Bead Solution tubes prior to extraction. Field tissue samples stored in 2 $\times$  CTAB were extracted by one of these methods: (i) the tissue was removed with sterile forceps, rinsed with sterile H<sub>2</sub>O and extracted with the UltraClean Plant DNA Isolation Kit as described above for dried samples; (ii) the tissue was removed with sterile forceps, rinsed with sterile H<sub>2</sub>O, and placed in 600  $\mu$ L of Nuclei Lysis Solution from the Wizard Genomic DNA Purification Kit, and extracted per the manufacturer's instructions (Promega Corp., Madison, Wisconsin). Cultures were grown on V8 media (20% w/v V8 juice, 0.1% w/v asparagine, 2.0% w/v maltose,

1.8% w/v agar). Approximately 2–4 mm of mycelium was aseptically excised from the growing edge of the colony and extracted with the UltraClean Plant DNA Isolation Kit as described above for dried herbarium samples. Extractions with evidence of co-extracted fungal pigments or polysaccharides were cleaned with the GeneClean Spin kit, according to the manufacturer's protocol (Qbiogene, Inc., Irvine, California).

*Polymerase chain reactions (PCR) and cycle sequencing.*—Unless otherwise noted, all PCRs were performed in 25  $\mu$ L reaction volumes with 12.5  $\mu$ L of PCR Master Mix (Promega Corp., Madison, Wisconsin), 1.25  $\mu$ L each of 10  $\mu$ M primers (upstream and downstream), and 10  $\mu$ L of diluted (10- to 100-fold) DNA template. PCR products were cleaned by one of two methods: (i) the majority were cleaned with Montage PCR Centrifugal Filter Devices (Millipore Corp., Billerica, Massachusetts) according to the manufacturer's protocol; (ii) if more than one PCR product was produced during amplification, then the band of the correct size was excised from a 1% agarose gel and cleaned with the MinElute PCR Gel Extraction Kit (Qiagen, Inc., Valencia, California). Cleaned PCR products were sequenced with BigDye Terminator sequencing enzyme v.3.1 (Applied Biosystems, Foster City, California) in the reaction: 2  $\mu$ L of diluted BigDye in a 1:3 dilution of BigDye:dilution buffer (400 mM Tris pH8.0, 10 mM MgCl<sub>2</sub>); 0.3  $\mu$ L of 10  $\mu$ M primer; 10–20 ng of cleaned PCR template; and H<sub>2</sub>O to 5  $\mu$ L total reaction volume. Cycle sequencing parameters consisted of a 2 min denaturation step at 94 C, then 35 cycles of 94 C for 39 s, 50 C for 15 s and 60 C for 4 min. Sequencing reactions were cleaned by ethanol precipitation and sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). All DNA sequences have been deposited in GenBank, Accessions AY916668–AY916757 (TABLE I). Specific primers and PCR amplification parameters for each gene region are provided below.

The first 1250 bp of the large ribosomal subunit (LSU) were amplified with primers LSU4-B (5'-CTGGACCGTG-TACAAGTCTCCTG, a basidiomycete-specific primer designed as the reverse-complement of the Gardes & Bruns [1993] primer ITS4-B) and LR6 and sequenced with LSU4-B, LR6, LR3 and LR3R (primer sequences for LR6, LR3 and LR3R are available on-line from the mycology lab of Duke University, <http://www.biology.duke.edu/fungi/mycolab/primers.htm>). Amplification was achieved with an initial denaturation step of 5 min at 94 C; 35 cycles of 30 s at 94 C, 45 s at 50 C, and 1 min at 72 C; and a final extension of 7 min at 72 C.

The complete small ribosomal subunit (SSU) of approximately 1800 bp was amplified with primers NS1 and NS8 and sequenced with NS1, NS2, NS3, NS4, NS5, NS6, NS7 and NS8 (<http://www.biology.duke.edu/fungi/mycolab/primers.htm>). Amplification and sequencing of the SSU from older herbarium specimens was achieved by two separate PCR reactions: (i) NS1 to NS4, sequenced with NS1, NS2, NS3 and NS4 and (ii) NS3 to NS8 sequenced with NS5, NS6, NS7 and NS8. In all cases,

SSU amplification was carried out under the same cycling program as for the LSU, except the primer annealing step was carried out at 55 C, and final extension was at 72 C for 10 min.

Primers ITS1-F and ITS4-B (Gardes and Bruns 1993) were used to amplify and sequence about 800 bp that make up the internal transcribed spacer (ITS) region consisting of ITS-1, 5.8S ribosomal DNA, and ITS-2. Amplification parameters were the same as for the LSU, except the cyclic extension step was shortened to 45 s.

To sequence the elongation factor 1- $\alpha$  region (EF1- $\alpha$ ), initially two degenerate primer pairs, (i) EF1-526F and 1567R and (ii) 983F and 2218R (Rehner and Buckley 2005), were used to amplify overlapping regions of the gene from a select group of exemplar taxa from the Marasmiaceae (*Marasmius* sp., *Crinipellis* sp. and *Campanella* sp.). The PCR products were ligated into pGEM-T Easy vectors and cloned in JM109 competent cells using the manufacturer's protocols (Promega Corp., Madison, Wisconsin). Transformed colonies were amplified directly with the M13/pUC 17-mer forward and reverse primers (MBI Fermentas, Hanover, Maryland) in the following manner: individual transformed colonies were selected with a sterile toothpick that was then agitated in the PCR cocktail described above except that 10  $\mu$ L of sterile H<sub>2</sub>O replaced the DNA template. The same cycling parameters as for the LSU were used, except 25 PCR amplification cycles were performed. PCR products of the correct size were cleaned and sequenced with the M13 primers. Sequences from exemplar taxa were aligned and several sets of primers were designed from nucleotide regions conserved across the exemplar taxa. After testing of primer pairs and PCR optimization, all additional taxa were amplified with primers RAS.EF1-F2 (5'-AGGARGCTGCTGAGYTSG) and RAS.EF1-R2 (5'-GCARG-MATCRCCVGAATTGACR), with the PCR cocktail initially described except primer concentration was 5  $\mu$ M instead of 10  $\mu$ M, and with these cycling parameters: initial denaturation for 5 min at 94 C; 40 cycles of 94 C for 30 s, 57 C for 1 min, and 72 C for 2 min; 72 C final extension for 7 min. The approximately 1200 bp products were sequenced with the amplification primers and two internal primers, 983F (Rehner and Buckley 2005) and EFgr (Steve Rehner pers comm).

Amplification and sequencing of the largest subunit of RNA polymerase II gene (RPB1) were achieved in a similar manner as for the EF1- $\alpha$ . Initially amplification and sequencing of exemplar taxa was done with primers RPB1-Af and RPB1-Cr (Matheny et al 2002), and internal sequencing primers RPB1-INT2F or RPB1-INT2.1F (P. Brandon Matheny, Clark University, Worcester, Massachusetts, pers comm). Optimized primers were designed and tested for enhanced specificity to marasmioid taxa, yielding a product of about 900 bp. All additional taxa were amplified and sequenced with primers RAS.RPB1-F2 (5'-CACCCACMACCCAATTTTCTGGGGG) and RAS.RPB1-R2 (5'-TCRTCCTCACTKCGCATYGCKCCWCCATCR), with one additional internal sequencing primer, RPB1-Br (Matheny et al 2002). Primer concentration in the PCR cocktail was 5  $\mu$ M instead of 10  $\mu$ M, and PCR cycling occurred as an initial denaturation step of 5 min at 94 C,

followed by 40 cycles of 30 s at 94 C, 1 min at 55 C and 2 min at 72 C and a final extension of 7 min at 72 C.

*Sampling strategy and sequence analyses.*—Preliminary phylogenetic analysis was conducted by analyzing LSU sequences of *Crinipellis pernicioso* and *Moniliophthora roreri* within the 877 taxa dataset of Moncalvo et al (2002). This dataset contains representatives of all major euagaric lineages, as well as exemplar taxa from the other major homobasidiomycete clades using a heterobasidiomycete as outgroup (Moncalvo et al 2002). Bootstrapping analyses using maximum parsimony were conducted in PAUP\* 4.0b10 (Swofford 2002) as described in Moncalvo et al (2002). Because these initial analyses could not resolve the generic placement of *C. pernicioso* and *M. roreri* within /marasmioid, additional taxon sampling focused on obtaining material and LSU sequences from taxa suspected to be members of the /marasmioid and /tetrapyrroid clades, including the type species and other representatives from all genera proposed to be members of /marasmioid as inferred from the classification of Singer (1976, 1986). Material from South America was preferentially sampled because both pathogens are found only on that continent. A total of 45 (39 /marasmioid, and 6 /tetrapyrroid as outgroup) LSU sequences were assembled and analyzed (Datamatrix A). Because phylogenetic placement of *M. roreri* and *C. pernicioso* could not be determined with confidence by the LSU dataset alone, Datamatrix B was constructed. Datamatrix B consisted of sequences of five gene regions (LSU, SSU, ITS, RPB1 and EF1- $\alpha$ ) for each of 15 taxa; these were selected as a cross sample representing each major clade uncovered with Datamatrix A.

Sequencing reactions were edited and contiguous sequences were assembled in Sequencher 4.1.4 (Gene Codes Corp., Ann Arbor, Michigan). Sequence alignments were constructed by eye in Se-Al v2.0a11 (Andrew Rambaut, Dept. Zoology, University of Oxford, UK; <http://evolve.zoo.ox.ac.uk/>). Sequences for Datamatrix A were trimmed to 1041 bp each, alignable across all bases. Datamatrix B consisted of a total of 5171 bp: 1769 bp of the SSU, alignable across all bases; 1041 bp from the LSU, alignable across all bases; ITS sequences were trimmed to 503 bp, alignable across all bases; RPB1 sequences were trimmed to 765 bp, alignable across all bases; and the EF1- $\alpha$  was trimmed to 1093 bp, of which 120 bp that were too variable to confidently align were excluded from the final analysis.

Maximum parsimony (MP) analyses were conducted in PAUP\* v4.0b10 as heuristic searches with 100 random addition replicates and TBR branch swapping. Support for the branching topologies was evaluated by bootstrap analysis derived from 1000 replicates with 10 random addition replicates each. Maximum likelihood (ML) analyses were conducted by the quartet puzzling method (Strimmer and von Haeseler 1996) in PAUP\* with 10 000 puzzling steps; transition/transversion ratio = 2. For both datamatrices, the /tetrapyrroid fungi were selected as outgroups for rooting purposes.

TABLE I. Marasmiaceae collections used in this study

Name	Origin	Speciman ID	Voucher <sup>a</sup>	LSU <sup>b</sup>	SSU <sup>b</sup>	ITS <sup>b</sup>	RPBI <sup>b</sup>	EFI-alpha <sup>b</sup>
<i>Ampyloflagellula inflata</i> Agerer & Boidin				AY570990 <sup>f</sup>		AY571027 <sup>f</sup>		
<i>Campanella</i> sp.				AF261340 <sup>d</sup>				
<i>Campanella</i> sp.	Pakaraima Mountains, Guyana	MCA 1689	BPI	AY916668	AY916669	AY916670	AY916671	AY916672&3
<i>Campanella</i> sp.	Pakaraima Mountains, Guyana	MCA 2235	BPI	AY916674	AY916675	AY916676	AY916677	AY916678
<i>Chaetocalathus biliputianus</i> (Mont.) Singer				AY570996 <sup>f</sup>				AY916679
<i>Chaetocalathus biliputianus</i>	Luquillo Mountains, Puerto Rico	MCA 485	BPI	AY916680	AY916681	AY916682	AY916683	
<i>Chaetocalathus</i> sp.				AF261347 <sup>d</sup>				
<i>Chaetocalathus</i> cf. <i>columellifer</i>	Bilsa, Ecuador	MCA 2538	BPI	AY916684	AY916685	AY916686	AY916687	AY916688
<i>Crinipellis campanella</i> (Pk.) Singer				U11916 <sup>e</sup>				
<i>Crinipellis maxima</i> A.H. Sm. & M.B. Walters				AF042630 <sup>c</sup>				
<i>Crinipellis setipes</i> (Pk.) Singer <sup>g</sup>	Montgomery Co., VA	RV 83/194	BPI	AY916689				
<i>Crinipellis stipitaria</i> (Fr.) Pat.				AY570997 <sup>f</sup>				
<i>Crinipellis zonata</i> (Pk.) Sacc.	Charlottesville, Virginia, USA	OKM 25450	VPI	AY916690	AY916691	AY916692	AY916693	AY916694
<i>Crinipellis</i> sp.				AF261348 <sup>d</sup>				
<i>Crinipellis</i> sp.	Pakaraima Mountains, Guyana	MCA 2240	BPI	AY916695				
<i>Crinipellis</i> sp.	Chantaburi Prov., Thailand	OKM 26890	VPI	AY916696	AY916697	AY916698		
<i>Crinipellis</i> sp.	Pakaraima Mountains, Guyana	MCA 1527	BPI	AY916699	AY916700	AY916701	AY916702	AY916703
<i>Marasmius</i> cf. <i>cladophyllus</i>	Pakaraima Mountains, Guyana	MCA 1837	BPI	AY916704		AY916705	AY916706	AY916707
<i>Marasmius delectans</i> Morgan				U11922 <sup>c</sup>				
<i>Marasmius plicatulus</i> Peck	Giles Co., Virginia, USA	MCA 1409	BPI	AY916708				
<i>Marasmius robula</i> (Scop.) Fr.				AF261345 <sup>d</sup>				
<i>Marasmius</i> sp. (pleurotoid)	Pakaraima Mountains, Guyana	MCA 1577	BPI	AY916709	AY916710	AY916711	AY916712	AY916713
<i>Marasmius</i> sp.				AF261342 <sup>d</sup>				
<i>Marasmius</i> sp.	Pakaraima Mountains, Guyana	MCA 1711	BPI	AY916714				
<i>Marasmius</i> sp.	Pakaraima Mountains, Guyana	MCA 1719	BPI	AY916715				
<i>Marasmius</i> sp.	Pakaraima Mountains, Guyana	MCA 2060	BPI	AY916716				
<i>Marasmius</i> sp.	Bilsa, Ecuador	MCA 2546	BPI	AY916717				
<i>Marasmius</i> sp.	Pakaraima Mountains, Guyana	MCA 1708	BPI	AY916718	AY916719	AY916720	AY916721	AY916722

TABLE I. Continued

Name	Origin	Speciman ID	Voucher <sup>a</sup>	LSU <sup>b</sup>	SSU <sup>b</sup>	ITS <sup>b</sup>	RPBI <sup>b</sup>	EF1-alpha <sup>b</sup>
<i>Marasmius</i> sp.	Pakaraima Mountains, Guyana	MCA 1611	BPI	AY916723	AY916724	AY916725	AY916726	AY916727
<i>Marasmius</i> sp.	Pakaraima Mountains, Guyana	MCA 1594	BPI	AY916728				
<i>Marasmius</i> sp.	near Quevedo, Ecuador	MCA 2525	BPI	AY916729				
<i>Marasmius</i> sp.	near Quevedo, Ecuador	MCA 2526	BPI	AY916730				
<i>Marasmius</i> sp.	Pakaraima Mountains, Guyana	MCA 1506	BPI	AY916731	AY916732	AY916733	AY916734	AY916735
<i>Marasmius</i> sp.	near Quevedo, Ecuador	MCA 2523	BPI	AY916736				
<i>Moniliophthora perniciosa</i> (Stahel)	Napo Prov., Ecuador	DIS70	CABI	AY916737				
Aime comb. nov.								
<i>Moniliophthora perniciosa</i>	Napo Prov., Ecuador	DIS71	CABI	AY916738	AY916739		AY916740	AY916741
<i>Moniliophthora perniciosa</i>	near Quevedo, Ecuador	MCA 2520	BPI	AY916742		AY916743		
<i>Moniliophthora roreri</i> (Cif.) H.C. Evans et al.	Turrialba, Costa Rica	C21	CATIE	AY916744	AY916745	AY916746	AY916747	AY916748
<i>Moniliophthora roreri</i>	Turrialba, Costa Rica	C22	CATIE	AY916749				
<i>Moniliophthora roreri</i>	near Quevedo, Ecuador	MCA 2521	BPI	AY916750				
<i>Moniliophthora</i> sp. (grass endophyte)	NM, USA	GJS 00-165	BPI	AY916751				
<i>Moniliophthora</i> sp. (grass endophyte)	NM, USA	MCA 2500	BPI	AY916752	AY916753	AY916754	AY916755	AY916756
<i>Tetrapyrgos subdendrophora</i> (Redhead) Horak <sup>h</sup>				AF042629 <sup>c</sup>				
<i>Tetrapyrgos</i> sp.	Pakaraima Mountains, Guyana	MCA 2162	BPI	AY916757				
<i>Tetrapyrgos</i> sp.				AF261338 <sup>d</sup>				

<sup>a</sup>Voucher: VPI = Massey Herbarium, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA; BPI = U.S. National Fungus Collections, Beltsville, MD, USA; CATIE = CATIE culture collection, Turrialba, Costa Rica with duplicate culture held at SBML; CABI = CABI Bioscience culture collection, U.K with duplicate culture held at SBML

<sup>b</sup> GenBank Accession number for gene

<sup>c</sup> Moncalvo et al. 2000

<sup>d</sup> Moncalvo et al. 2002

<sup>e</sup> Chapela et al. 1994

<sup>f</sup> Bodensteiner et al. 2004

<sup>g</sup> originally as *Crinipellis stipitaria*

<sup>h</sup> as *Campanella subdendrophora* Redhead

## RESULTS

Preliminary analyses based on the dataset of Moncalvo et al (2002), placed *M. roreri* and *C. pernicioso* within the /marasmiaceae sensu Moncalvo et al (2002) (results not shown, but available from the lead author upon request); within the /marasmiaceae these taxa are strongly supported (67% bootstrap support) members of the /marasmioid with /tetrapyrgoid as the sister clade. In these analyses, *M. roreri* and *C. pernicioso* appear as sister taxa, which together are sister to the genus *Chaetocalathus*, and are more distantly related to the genera *Crinipellis* and *Marasmius*.

Combined analyses of Datamatrix A, based on sequence data from the nLSU rDNA region alone, are shown (FIG. 2). Of 1041 included characters, 137 were parsimony-informative; 35 variable characters were parsimony-uninformative. Twenty-eight equally parsimonious trees of length 405 were found by MP; CI = 0.56; RI = 0.80. The same major clades were uncovered by both MP and ML, i.e. six monophyletic groups consisting of: (i) *Campanella* spp. and (ii) *Tetrapyrgos* spp. as outgroups; and (iii) *Crinipellis* spp. excepting *C. pernicioso*, hereinafter referred to as *Crinipellis* sensu stricto (s.s.); (iv) *Chaetocalathus* spp.; (v) *Marasmius* spp. (including *Amyloflagellula inflata*); and (vi) a group consisting of *M. roreri*, *C. pernicioso*, and an unnamed grass endophyte from New Mexico, hereinafter referred to as the *Moniliophthora* clade (FIG. 2). However, overall support for several of these groupings was low: only the genera *Marasmius*, *Crinipellis* s.s. and the two outgroup genera (*Campanella* and *Tetrapyrgos*) receive modest support with both methods; the *Moniliophthora* clade is not supported by bootstrapping methods, and weakly supported (45%) by quartet puzzling (FIG. 2).

Because Datamatrix A (LSU sequences alone) could not resolve the generic or inter-specific relationships of *M. roreri* and *C. pernicioso* with confidence, Datamatrix B was constructed. A shaded circle in FIG. 2 indicates exemplar taxa selected for additional sequencing to construct Datamatrix B. Results of analyses with Datamatrix B are presented (FIG. 3). Of 5049 included characters, 630 were parsimony-informative and 318 variable characters were not parsimony-informative. A single MP tree was found of length = 2044, CI = 0.62, and RI = 0.54. Both MP and ML uncovered the same clades in the analyses of Datamatrix B as had been uncovered with Datamatrix A. Significant support for each of the four ingroup taxa (*Crinipellis* s.s., *Marasmius*, *Chaetocalathus*, and the *Moniliophthora* clade) was found by both bootstrapping and quartet puzzling with Datamatrix B (FIG. 3).

## DISCUSSION

Subtribus Crinipellinae Singer (Tricholomataceae R. Heim ex Pouzar, tribus Marasmiaceae Fayod ex Schröter) contains those marasmioid agarics with amyloid or dextrinoid epicuticular hairs or appendages (Singer 1976, 1986). Genera allied in the Crinipellinae are *Crinipellis*, *Chaetocalathus*, *Amyloflagellula* Singer, *Lachnella* Fr., and *Flagelloscypha* Donk apud Singer (Singer 1986). The majority of these species are saprotrophic litter fungi, and as a group considered to be sister to *Marasmius* and eight other reduced marasmioid genera (Singer 1986). Prior work has shown that *Lachnella* (Moncalvo et al 2002) and *Flagelloscypha* (Bodensteiner et al 2004) do not share a common ancestry with the core Crinipellinae. Our results (FIG. 2) and those of Bodensteiner et al (2004) have shown that at least some elements of *Amyloflagellula* are congeneric with *Marasmius* s.l. in /marasmioid. Furthermore, Moncalvo et al (2002) showed strong support for a monophyletic group consisting of *Marasmius*, *Chaetocalathus* and *Crinipellis*, termed /marasmioid, that is sister to *Tetrapyrgos* E. Horak and *Campanella* Henn. within a larger /marasmiaceae. Presently the Marasmiaceae Kühner is being redefined, and will incorporate those fungi in /marasmioid, i.e. *Marasmius*, *Crinipellis*, and *Chaetocalathus* (Dennis Desjardin, San Francisco State University and Andy Wilson, Clark University, pers comm). The present work shows that the two cacao pathogens also belong to the Marasmiaceae s.s. (FIGS. 2 and 3).

*Moniliophthora* was described as a monotypic, anamorphic genus of basidiomycetes, *incertae sedis*, although similarities in disease symptomatology between *M. roreri* and *C. pernicioso* had been noted (Evans et al 1978). Citing these symptomatological similarities and other similarities in cytology, Evans et al (2002) transferred *M. roreri* to *Crinipellis*, although no other species of the genus *Crinipellis* were examined. The present study shows that *Moniliophthora* is a member of the Marasmiaceae s.s., and that *M. roreri* and *C. pernicioso* are closely related fungi. Within the confines of our sampling, *M. roreri* and *C. pernicioso* appear as sister species (FIG. 3). Neither, however, is congeneric with *Crinipellis* as exemplified by the type species, *C. stipitaria* (Fr.) Pat., (Singer 1942, 1986; Kühner 1980) (FIG. 2). Rather, the two cacao pathogens form part of a distinct lineage within the Marasmiaceae s.s. that is not congeneric with any other genus previously allied within Subtribus Crinipellinae.

We recognize that changing the names of well-known pathogens is often accepted with reluctance. Singer noted in 1976 that it took more than 30 years

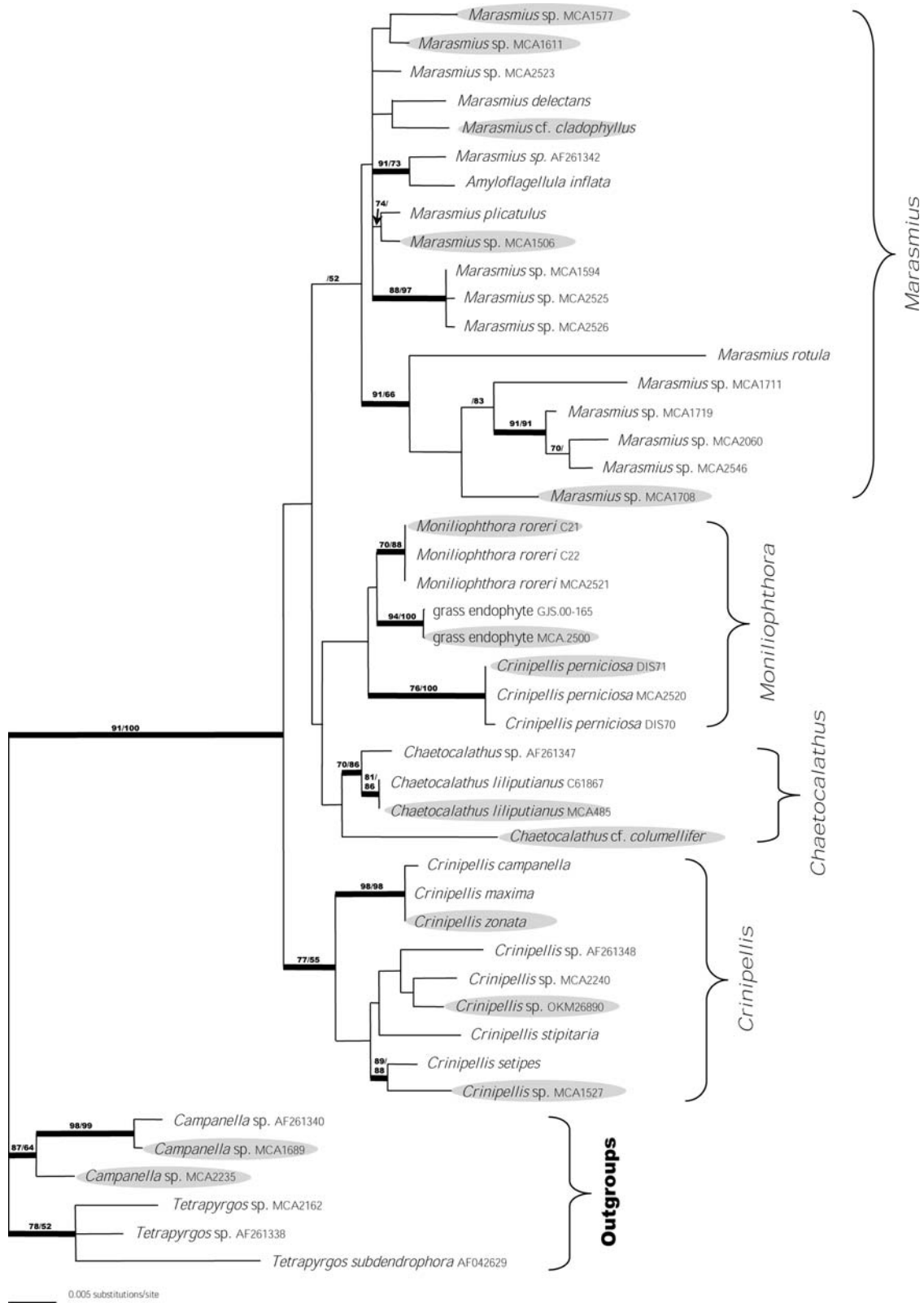


FIG. 2. Rooted quartet puzzling tree produced with nuclear large subunit ribosomal DNA sequences of Marasmiaceae s.s. taxa. Thickened branches are those receiving significant ( $\geq 70\%$  quartet puzzling,  $\geq 50\%$  bootstrapping) support by both ML and MP methods. Numbers above branches give support values: the first number is by quartet puzzling, the number after the slash is bootstrapping support. Taxa selected for additional gene sequencing to construct Datamatrix B are indicated by shaded gray ovals.

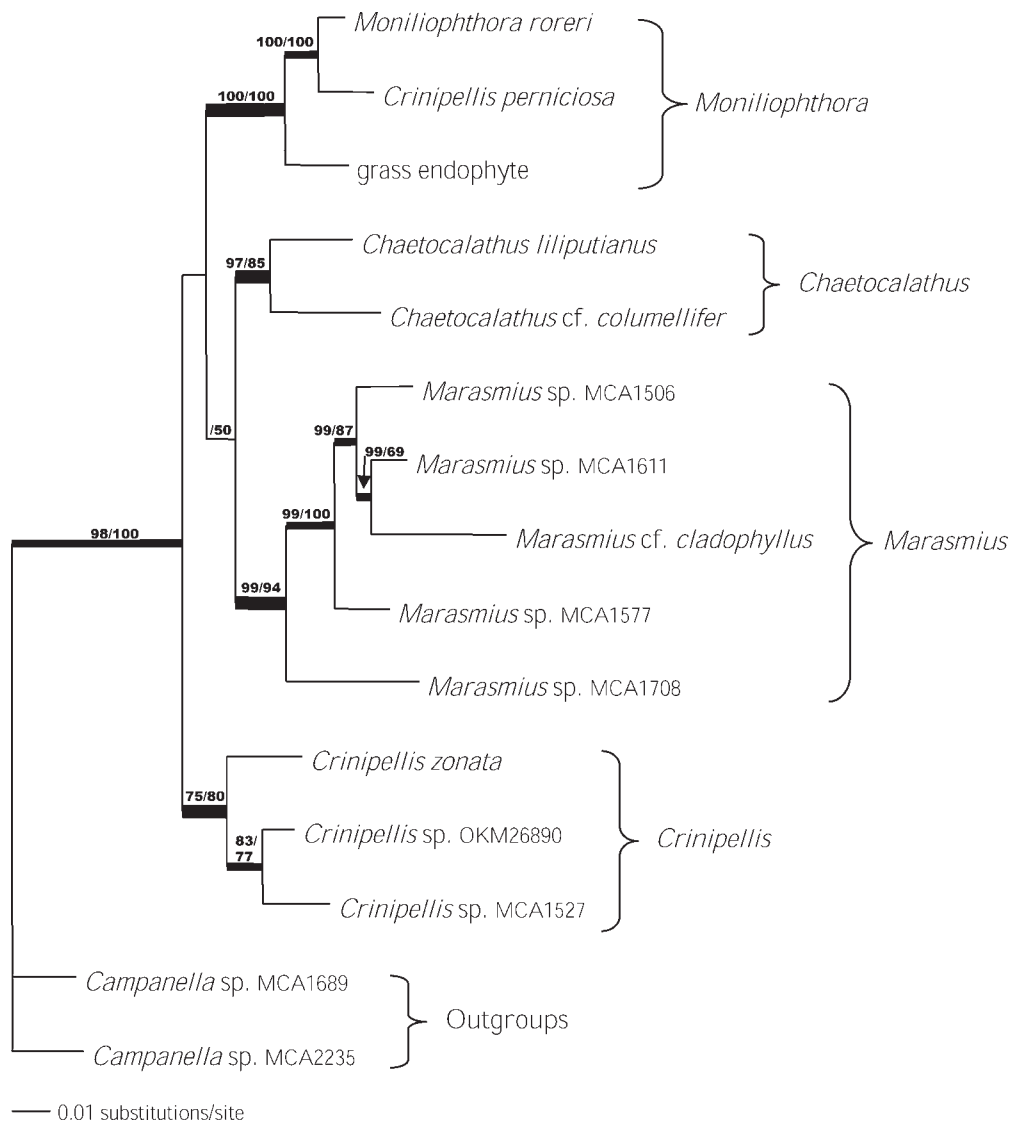


FIG. 3. Rooted quartet puzzling tree produced with combined sequences of five gene regions (nuclear large subunit ribosomal DNA, nuclear small subunit ribosomal DNA, internal transcribed spacer region, elongation factor 1- $\alpha$ , and the largest subunit of RNA polymerase II) of select Marasmiaceae s.s. taxa. Thickened branches are those receiving significant ( $\geq 70\%$  quartet puzzling,  $\geq 50\%$  bootstrapping) support by both ML and MP methods. Numbers above branches give support values: the first number is by quartet puzzling, the number after the slash is bootstrapping support.

for the preferential use of the name *C. perniciosus* over its prior name of *Marasmius perniciosus*; the name *Monilia roreri* still is often used for the causal agent of frosty pod rot even though 25 years have passed since the transfer of that taxon into *Moniliophthora* (Evans et al 2002). Nevertheless the present study makes necessary a taxonomic re-evaluation of the cacao pathogens.

At present two options are available for naming and identifying the monophyletic clade that contains *M. roreri* and *C. perniciosus*: (i) A new generic name is erected; or (ii) the name *Moniliophthora*, although typified by an anamorphic fungus, is used in essence

as a pleomorphic name, and *C. perniciosus* is transferred into that genus. We have chosen the second option for several reasons. First, we advocate that nomenclature should reflect best phylogenetic estimates, and furthermore, that the proliferation of names in the literature should be avoided where at all possible. If a new generic name is erected to accommodate the agent of witches' broom, then the current International Code of Botanical Nomenclature (ICBN) (Greuter et al 1999) does not allow the simultaneous transfer of *M. roreri* into that genus (Paul Kirk, CABI Bioscience, UK, pers comm), resulting in a situation where two sister-species do

not share the same generic name. Although it is almost never done, to the best of our understanding nothing within the ICBN prohibits the transfer of a teleomorphic fungus into a genus based on an anamorph. Secondly, Evans et al (2002, 2003) provide cytological evidence that *M. roleri* is, in fact, a teleomorphic fungus that undergoes meiosis within its “conidia.” Therefore the name *Moniliophthora* could be considered the earliest legitimate name for this genus.

The genus *Crinipellis* is delimited as those centrally-stipitate agarics of marasmioid stature that possess dextrinoid “hairs” on the pileipellis (Singer 1942). Approximately 75 species are currently recognized in the genus (Kirk et al 2001), the majority of which are saprotrophic litter and wood decomposers (Holliday 1980, Singer 1986). Eleven of these, including *C. perniciosus* and *C. eggersii* Pat., were placed in Section *Iopodinae* Singer (Singer 1976, 1986). We have molecular evidence that *C. eggersii* also belongs in *Moniliophthora* (unpublished), and it is likely that, pending molecular and type studies, the entirety of *Crinipellis* Section *Iopodinae* will be found to be congeneric with *Moniliophthora*. Section *Iopodinae* contains the other parasitic members of *Crinipellis*; other taxa in this section infect tropical rainforest and greenhouse trees such as species of *Siparuna* Aubl. and *Vitex* L (Singer 1976). Additionally, mushrooms in Section *Iopodinae* share the features of purple, violet or red pigments in the pileus that do not change color in the presence of an alkaloid solution, whereas members of the other sections of *Crinipellis* are brown pigmented or if not, the pigments turn green in the presence of KOH (Singer 1976, 1986). A few plant pathogenic species currently placed in the genus *Marasmiellus* Murrill—a genus now known to be synonymous with *Gymnopus* (Pers.) Roussel (Mata et al 2004)—have setose hairs on the pileus and may also belong to *Moniliophthora*.

It is tempting to speculate on the origins of *Moniliophthora*, especially given the discovery that an unnamed asymptomatic and presumably symbiotic endophyte of grasses, isolated from New Mexico, is congeneric with the two pathogens (FIG. 3), and it might be that the pathogens have evolved from a common biotrophic ancestor somewhere near the Andes of South America as commonly conjectured (see Evans et al 2003). Certainly, other potentially endophytic, biotrophic species of *Moniliophthora* await discovery.

#### TAXONOMY

***Moniliophthora perniciosus*** (Stahel) Aime & Phillips-Mora, comb. nov. FIGS. 1a, b

≡ *Marasmius perniciosus* Stahel, Dep. Landb. Suriname 33:16. 1915.

≡ *Crinipellis perniciosus* (Stahel) Singer, Lilloa 8:503. 1943 [1942].

This taxon is completely described in Singer (1942, 1976) and Holliday (1970) and discussed in Evans and Barreto (1996).

#### ACKNOWLEDGMENTS

First and foremost we are grateful to Cindy Park for excellent laboratory assistance; Dr Harry Evans for laying the groundwork for, and Drs Gary Samuels and Prakash Hebbar encouraging the present study. We thank Dr Dennis Desjardin for discussing the current taxonomic status of the Marasmiaceae and are indebted to Drs David Hawksworth, Paul Kirk and Walter Gams for elucidating details of the ICBN. Dr Manfred Binder generously shared DNAs of three crucial taxa, and Drs Steve Rehner and Brandon Matheny shared unpublished primer sequences. We thank Dr Jerry Barrow for sending a culture of an unknown grass endophyte that turned out to be another species of *Moniliophthora*. Finally, we thank Dr Amy Rossman for critically reviewing an earlier version of this manuscript and Dr Scott Redhead for invaluable critiques, suggestions, and discussion.

#### LITERATURE CITED

- Bodensteiner P, Binder M, Moncalvo JM, Agerer R, Hibbett DS. 2004. Phylogenetic relationships of cypheloid homobasidiomycetes. *Molec Phylo Evol* 33:501–515.
- Chapela IH, Rehner SA, Schultz TR, Mueller UG. 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science* 266:1691–1694.
- Ciferri R, Parodi E. 1933. Descrizione del fungo che causa la “Moniliasi” del cacao. *Phytopathologische Zeitschrift* 5:539–542.
- Evans HC. 1981. Pod rot of cacao caused by *Moniliophthora (Monilia) roleri*. *Phytopath Pap* 24:1–44.
- . 1986. A reassessment of *Moniliophthora (Monilia)* pod rot of cocoa. *Cocoa Growers' Bulletin* 37:34–43.
- , Barreto RW. 1996. *Crinipellis perniciosus*: a much investigated but little understood fungus. *Mycologist* 10:58–61.
- , Holmes KA, Phillips W, Wilkinson MJ. 2002. What's in a name: *Crinipellis*, the final resting place for the frosty pod rot pathogen of cocoa? *Mycologist* 16:148–152.
- , ———, Reid AP. 2003. Phylogeny of the frosty pod rot pathogen of cocoa. *Plant Path* 52:476–485.
- , Stalpers JA, Samson RA, Benny GL. 1978. On the taxonomy of *Monilia roleri*, an important pathogen of *Theobroma cacao* in South America. *Can J Bot* 56:2528–2532.
- Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes—application to the iden-

- tification of mycorrhizae and rusts. *Molec Ecol* 2:113–118.
- Greuter W, McNeill J, Barrie FR, Burdet HM, Demoulin V, Filgueiras TS, Nicolson DH, Silva PC, Skog JE, Trehane P, Turland NJ, Hawksworth DL, eds. 1999. International Code of Botanical Nomenclature (St Louis Code). *Regnum Vegetabile* 138. Königstein, Germany: Koeltz Scientific Books.
- Griffith GW, Nicholson J, Nenninger A, Birch RN, Hedger JN. 2003. Witches' brooms and frosty pods: two major pathogens of cacao. *New Zealand J Bot* 41:423–435.
- Hillis DM, Moritz C, Mable BK. 1996. *Molecular Systematics*. 2nd ed. Sunderland, Massachusetts: Sinauer Associates. 655 p.
- Holliday P. 1970. *Crinipellis perniciosa*. CMI Descriptions of Pathogenic Fungi and Bacteria No. 223.
- . 1980. *Fungus Diseases of Tropical Crops*. New York: Dover Publications, Inc. 607 p.
- Isaac S, Hardwick K, Collin H. 1993. Interactions between the pathogen *Crinipellis perniciosa* and cocoa tissue. In: Isaac S, Frankland JC, Watling R, and Whalley AJS, eds. *Aspects of tropical mycology*. Cambridge, UK: Cambridge University Press. p 219–232.
- Kirk PM, Cannon PF, David JC, Stalpers JA. 2001. *Dictionary of the fungi*. Oxon, UK: CAB International. 655 p.
- Kühner R. 1980. *Les Hyménomycètes agaricoïdes (Agaricales, Tricholomatales, Pluteales, Russulales) Etude générale et classification*. Bulletin de la Société Linnéenne de Lyon, France. 1027 p.
- Mata JL, Hughes KW, Petersen RH. 2004. Phylogenetic placement of *Marasmiellus juniperinus*. *Mycoscience* 45:214–221.
- Matheny PB, Liu YJ, Ammirati JF, Hall BD. 2002. Using RPB1 sequences to improve phylogenetic inference among mushrooms (*Inocybe*, Agaricales). *Amer J Bot* 89:688–698.
- Moncalvo JM, Lutzoni FM, Rehner SA, Johnson J, Vilgalys R. 2000. Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *Syst Biol* 49:278–305.
- , Vilgalys R, Redhead SA, Johnson JE, James TY, Aime MC, Hofstetter V, Verduin SJW, Larsson E, Baroni TJ, Thorn RG, Jacobsson S, Cléménçon H, Miller OK. 2002. One hundred and seventeen clades of euagarics. *Molec Phylo Evol* 23:357–400.
- Pereira JL. 1999. Renewed advance of witches' broom disease of cocoa: 100 years later. In: International Cocoa Research Conference, 12th, Salvador, Bahia, Brasil, 1996. *Proceedings*. Lagos, Nigeria: Cocoa Producers' Alliance: 87–91.
- , Ram A, Figueiredo JM, Almeida LCC. 1990. The first occurrence of witches' broom disease in the principal cocoa growing region of Brazil. *Tropical Agriculture* 67:188–189.
- Phillips-Mora W. 2003. Origin, biogeography, genetic diversity and taxonomic affinities of the cacao fungus *Moniliophthora roreri* as determined using molecular, phytopathological and morpho-physiological evidence [Doctoral dissertation]. Reading, UK: Univ. Reading. 349 p.
- , Krauss U, Evans H, Wilkinson M. 2003. Genetic diversity of the cacao pathogen *Moniliophthora roreri* (Cif.) Evans et al. in Tropical America. In: *Proceedings of the Fourteenth International Cocoa Research Conference*. Accra, Ghana: Cocoa Producers' Alliance.
- Rehner SA, Buckley E. 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1- $\alpha$  sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. *Mycologia* 97:84–98.
- Singer R. 1942. A monographic study of the genera "Crinipellis" and "Chaetocalathus." *Lilloa* 8:441–534.
- . 1976. *Marasmiaceae (Basidiomycetes-Tricholomataceae)*. *Flora Neotropica* No. 17. New York: New York Botanical Garden. 347 p.
- . 1986. *The Agaricales in Modern Taxonomy*. Koeltz Scientific Books, Germany. 981 p.
- Stahel G. 1915. *Marasmius perniciosus* nov. spec. *Dept Landbouw in Suriname Bull* 33:1–27.
- Strimmer K, von Haeseler A. 1996. Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Mol Biol Evol* 13:964–969.
- Swofford DL. 2002. *PAUP\**. Phylogenetic analysis using parsimony (\*and other methods), version 4. Sunderland, Massachusetts: Sinauer Associates.